

**Dihydroartemisinin is a hypoxia active  
anticancer drug that improves the cytotoxic  
action of radiotherapy**

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*For my grandmother*

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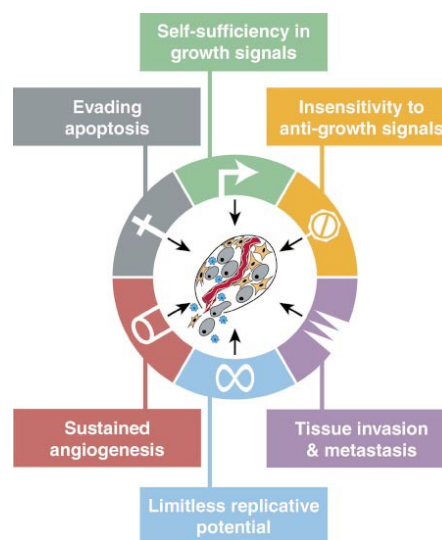
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## 1 Introduction

### 1.1 Hallmarks of cancer and chemotherapy resistance

Tumorigenesis is a multistep process during which genetic alterations occur that could drive to a progressive transformation of normal human cells into highly malignant cells. There are different types of cancer in human population and they mostly have an age dependent incidence (Renan 1993). Most of the cancers could be characterized by six essential alterations in cell physiology that correlate in malignant tumor growth. These alterations are listed in Fig. 1 (Hanahan and Weinberg 2000).



**Fig. 1 Acquired capabilities of cancer (adapted from (Hanahan and Weinberg 2000)).** The tumor cells are characterized by their uncontrolled proliferative potential and their ability to invade tissues and to metastasize. Cancer cells have developed specific molecular mechanisms in order to avoid growth inhibition and apoptosis. In addition, they stimulate their own growth by auto- and paracrine signals and the induction of angiogenesis.

Tumor cells are able to use their own signals and behave independently from their normal tissue microenvironment. The overexpression of specific receptors could lead to a high response of cancer cells to the environmental levels of growth factors (GF) that under normal conditions would not lead to proliferation (Hanahan and Weinberg 2000). In addition cancer cells are using for their survival the PI3 kinase pathway as a downstream factors (Downward 1998). PI3 kinase pathway normally regulates cellular processes such as cell growth, survival, differentiation, chemotaxis and metabolism (Workman, Clarke et al. 2006). Loss of PTEN phosphatase as well as overexpression of the upstream receptor of tyrosine kinases and downstream serine/threonine

kinase PKB/Akt, are highly associated with tumorigenesis (Vivanco and Sawyers 2002). Therefore PI3 kinases inhibitors as well as drugs targeting other members of the pathway, for example PKB/Akt would be pivotal to cure those tumors. Some of the known inhibitors are: LY294002 and wortmanin (Yaguchi, Fukui et al. 2006).

Another characteristic of the tumor cells is the resistance toward apoptosis induction. The p53 tumor suppressor protein can control the apoptosis by upregulating pro-apoptotic protein Bax in response to DNA damage. Bax leads to the mitochondrial apoptotic pathway activation (Hanahan and Weinberg 2000). Loss via mutations of p53 is common in cancer cells and it is observed in 50% of human cancers, that result in the removal of the key regulator of apoptosis in response to DNA damage (Harris 1996) and leads to therapy resistance.

## 1.2 Microenvironment of solid tumors

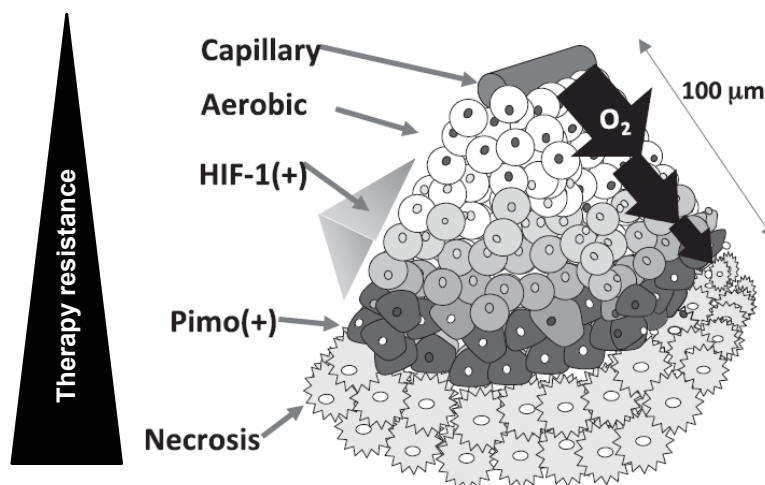
Most of the solid tumors are characterized of regions of acute and chronic hypoxia or even anoxia that correspond to a poor clinical prognosis for the cancer patients (Fig. 2). Under normoxic conditions, ionizing radiation produces DNA damage such as DNA double strand breaks. Radio-sensitization requires the presence of O<sub>2</sub> at the time of ionizing radiation in order to make permanent and irreparable lethal DSBs produced by free radicals. Therefore, under hypoxic conditions the number of double strand breaks are 2-3 fold decreased (Frankenberg-Schwager, Frankenberg et al. 1991; Brown, Evans et al. 1992; Wang, Biedermann et al. 1992; Terris, Ho et al. 2002; Olive and Banath 2004; Bristow and Hill 2008). Genetic instability that is caused by hypoxia can arise as a function to hypoxia-mediated resistance to apoptosis and decrease of DNA repair (Moulder and Rockwell 1987; Li, Little et al. 2001; Harris 2002). This contributes to the resistance of these hypoxic tumors to classical chemotherapy and radiotherapy.

Hypoxia inducible factor HIF-1 plays a key role in response to cellular hypoxia. HIF-1 consists of two subunits, the oxygen sensitive HIF-1 $\alpha$  and the constitutively expressed HIF-1 $\beta$  (Wang, Jiang et al. 1995). Under normoxic conditions, the von Hippel-Lindau tumor suppressor (pVHL), which is the recognition component of an E3 ubiquitin ligase complex targets HIF-1 $\alpha$ , leading to its ubiquitylation and subsequent proteosomal degradation (Iwai, Yamanaka et al. 1999; Lisztwan, Imbert et al. 1999; Cockman, Masson



et al. 2000; Kamura, Sato et al. 2000; Ohh, Park et al. 2000; Tanimoto, Makino et al. 2000). Under hypoxic conditions, the proline hydroxylases cannot be active so that proline residues are not hydroxylated, resulting in an accumulation of HIF-1 $\alpha$ . HIF-1 $\alpha$  then translocates to the nucleus where it dimerises with the subunit HIF-1 $\beta$ . HIF-1 recruits cofactors such as CBP/P300 for its full activation and translation of the target genes such as vascular endothelial growth factor (VEGF) (Forsythe, Jiang et al. 1996; Yamashita, Discher et al. 2001) (Fig.3).

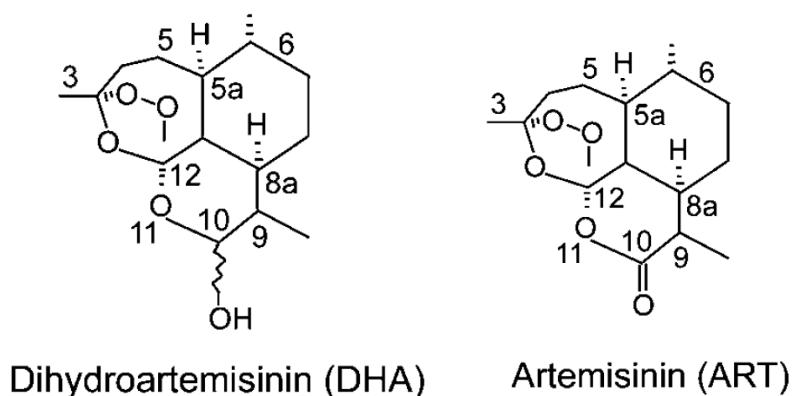
In cancer, both hypoxia and mutations in oncogenes and tumor suppressor genes increase HIF activity. HIF-1 $\alpha$  was found to be highly expressed in solid tumors such as colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate and renal carcinomas (Zhong, De Marzo et al. 1999). HIF-1 target genes have been identified, and many of these are associated with angiogenesis, invasion, and metastasis (Semenza 2003). Therefore high levels of HIF-1 $\alpha$  expression correlate with poor prognosis in tumor therapy making it potential target for anticancer drug development. A number of drugs that inhibit HIF-1 $\alpha$  have been redeveloped, however, none of them directly and specially target HIF-1 (Giaccia, Siim et al. 2003; Semenza 2003; Shalini and Bansal 2006).



**Fig. 2 Solid tumor microenvironment.** Solid tumors are characterized of regions with hypoxia and necrotic regions. Hypoxic cells are able to survive, however, they are protected from chemotherapy. Modified from (Kizaka-Kondoh, Kuchimaru et al. 2011).

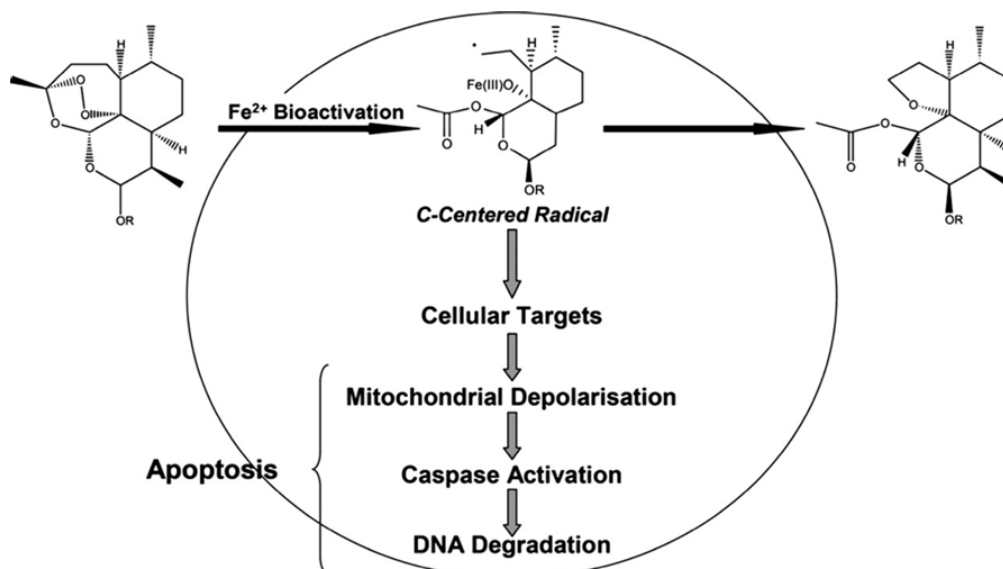


2004). The chemical structures of artemisinin and its derivative dihydroartemisinin are listed in Fig. 4.



**Fig. 4 Chemical structure of artemisinin and dihydroartemisinin (Hou, Wang et al. 2008)**

It is hypothesized, that ART reacts with Fe(II) species to form toxic carbon-centered radicals (O'Neill and Posner 2004) (Fig. 5). Iron is required for bioactivation of the endoperoxide bridge and is essential for the cytotoxicity of the drug (Beekman, Barentsen et al. 1997; Beekman, Wierenga et al. 1998). Interestingly, the cell lines which are the most sensitive to the drug, are characterized by high iron uptake, an effect that correlates with high proliferation of tumor cells (Kwok and Richardson 2002; Disbrow, Baege et al. 2005). Endoperoxide mediated cytotoxicity in cancer cells proceeds via apoptosis induction in caspase dependent manner, although the exact molecular basis of this induction has not been explained yet (Li, Shan et al. 2001; Sadava, Phillips et al. 2002; Wu, Zhou et al. 2004; Disbrow, Baege et al. 2005). Dihydroartemisinin has been reported to be an effective and water soluble analog (Janse, Waters et al. 1994; Tu 1999).

**Proposed chemical and molecular pathways of endoperoxide-induced HL-60 cell death.**

**Fig. 5 Proposed mechanism of endoperoxides action (Mercer, Maggs et al. 2007)** Intracellular free iron plays a major role in the activation of ART endoperoxides. Endoperoxides induce concentration and time-dependent apoptosis via mitochondrial membrane depolarization, caspase activation, and DNA degradation.

## 1.4 Mechanisms of death

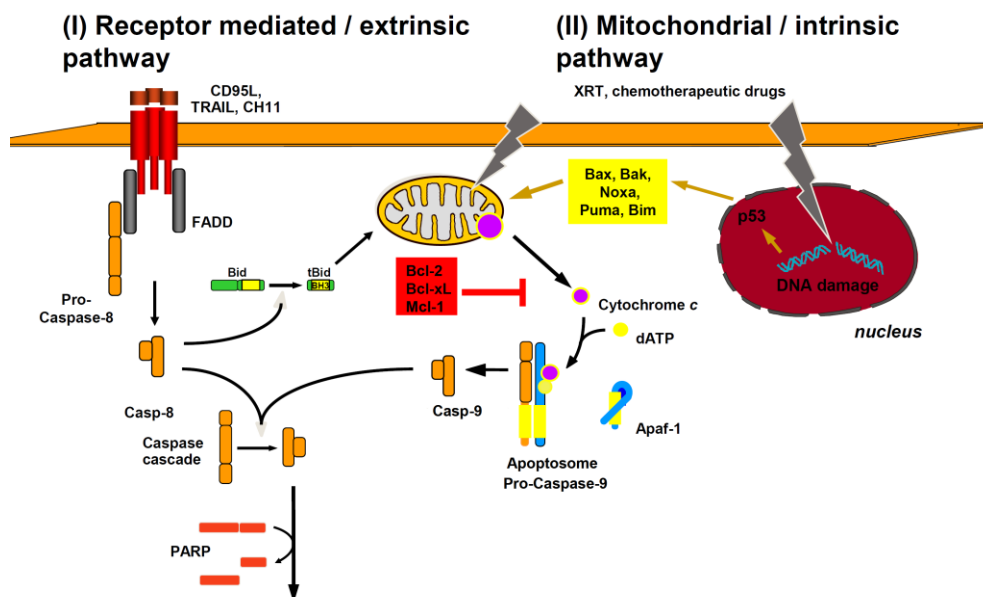
Although much is known about apoptosis, therapy-induced cell death has been reported to involve other death pathways as potential targets of therapy. Understanding the differences between the cell death pathway defects in each type of cancer, may offer a more tailored approach to choose specific and efficient treatments in order to enhance cell death (Speirs, Hwang et al. 2011). Besides the classical death pathways such as apoptosis, cell death can be induced by necrosis, autophagy and cellular senescence.

### 1.4.1 Apoptosis

The induction of apoptosis originates by two alternative pathways: one is mediated by the so called death receptor “extrinsic pathway” and the other pathway critically involves the mitochondria and is known as “intrinsic pathway”. Both pathways specially lead to the activation of caspases as critical apoptosis effectors. Active caspases cleave cellular substrates, leading to morphological changes characteristic of apoptosis (Krammer 1999; Schmitz, Kirchhoff et al. 2000).

Death receptors family (CD95R, TRAIL – R1, TRAIL-R2), is activated by the binding of ligands such as CD95L, TRAIL (TNF related apoptosis-inducing ligand) and leads to the formation of the DISC (death-inducing signaling complex). That results in caspase-8 activation via recruitment of DISC by the FAS-associated death domain protein (FADD). Active caspase-8 cleaves the effector caspase – 3 either directly or indirectly via mitochondrial apoptosis pathway (Ashkenazi 2002).

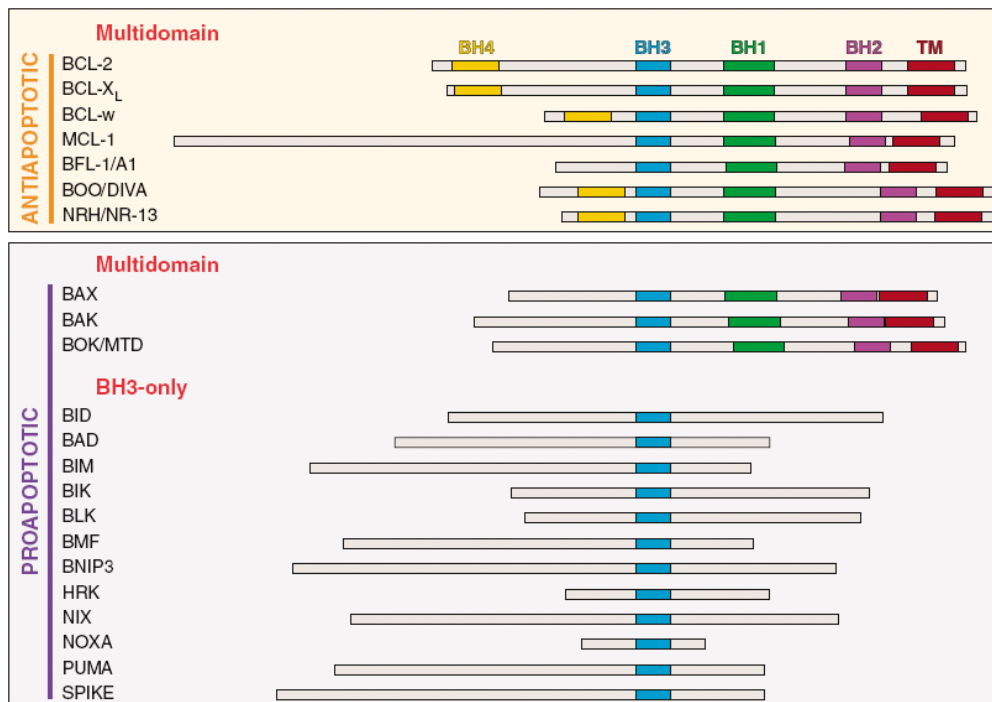
In the mitochondrial apoptosis pathway, downstream effector caspases are activated upon release into the cytosol of apoptogenic factors, such as cytochrome c, from the mitochondrial inner membrane space. Cytochrome c release triggers caspase-3 activation via formation of apoptosome (Apaf1, cytochrome-c and caspase 9) (Saelens, Festjens et al. 2004). Both pathways are represented in Fig. 6.



**Fig. 6 The two main apoptotic signaling pathways.** During apoptosis signaling through death receptors pathway, death ligands such as CD95L bind to their receptors and lead to the formation of the death-inducing signaling complex (DISC). The initiator procaspase 8 is recruited to the DISC by FADD (FAS-associated death domain protein). Caspase 8 is activated by autocatalytic cleavage. Chemotherapy, irradiation and other stimuli can induce apoptosis via mitochondrial pathway. Bcl-2 protein family plays essential role in this pathway. Pro-apoptotic proteins like BAX, BID, BAD and BIM are important mediators of apoptosis signals. Activation of the mitochondria leads to the cytochrome c release into the cytosol. Cytochrome c binds APAF-1 (apoptotic protease activating factor 1) to form apoptosome. At the apoptosome, initiator caspase 9 is activated. Both intrinsic and extrinsic apoptotic pathways lead to activation to the downstream effector caspases and inactivation of PARP (Chinnaiyan, O'Rourke et al. 1995)

### 1.4.2 Bcl-2 protein family

Bcl-2 proteins are essential in regulating death pathways at the mitochondrial level. Bcl-2 members can be divided into three subfamilies: the anti-apoptotic (Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, Mcl-1), the pro-apoptotic multidomain such as Bax, Bak and Bok/Mtd and the pro-apoptotic BH3-only subfamily listed in Fig 7 (Huang and Strasser 2000; Puthalakath and Strasser 2002; Borner 2003). The survival proteins such as, Bcl-2 and Bcl-X<sub>L</sub>, share three to four conserved Bcl-2 homology domains and are named “multidomain anti-apoptotic” members. The executioner proteins such as Bax and Bak share three conserved domains and are known as “multidomain pro-apoptotic” proteins. The “BH3-only” members share only the BH3 domain and function as sensors of cellular stress (Cory and Adams 2002; Petros, Olejniczak et al. 2004).



**Fig. 7 BCL-2 family proteins are structurally defined by their BCL-2 homology domains (Walensky 2006).** Bcl-2 proteins are subdivided either to pro- or anti-apoptotic and could be further subdivided into three groups. The antiapoptotic Bcl-2 subfamily proteins possess four short Bcl-2 homology (BH) domains and a C-terminal transmembrane (TM) domain. The pro-apoptotic multidomain such as Bax, Bak and Bok/Mtd are characterized by three BH domains and a hydrophobic TM domain. Finally the pro-apoptotic BH3-only subfamily, consist of only a BH3 domain.

The network of interactions among Bcl-2 family members is complex and remains a focus of recent investigation. The ability of anti-apoptotic proteins to form heterodimers with multidomain pro-apoptotic proteins suggests that

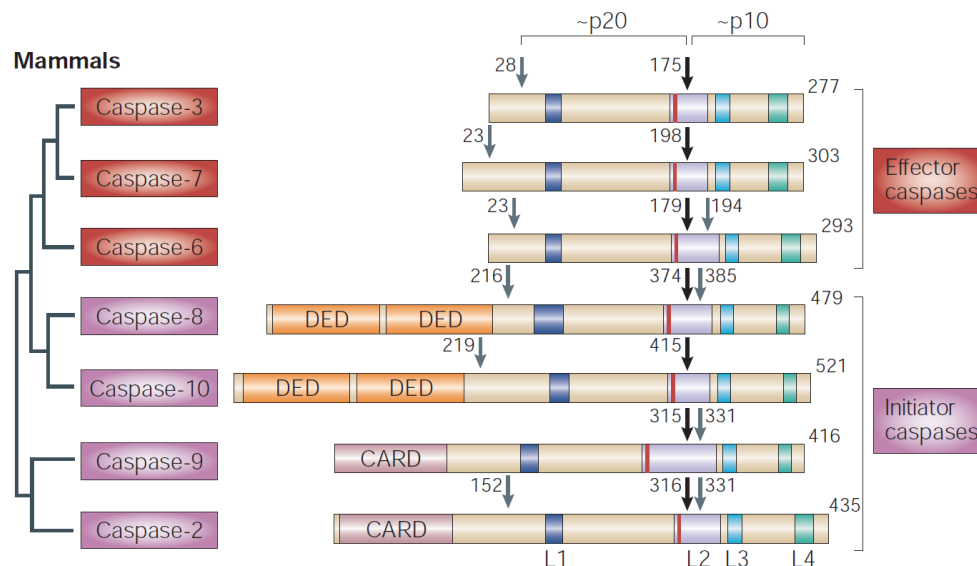
neutralizing competition plays an important role in the suppression of cell death (Oltvai, Millman et al. 1993; Sedlak, Oltvai et al. 1995; Diaz, Oltersdorf et al. 1997; Sattler, Liang et al. 1997; Gross, Jockel et al. 1998; Wang, Gross et al. 1998; Suzuki, Youle et al. 2000).

### 1.4.3 Caspases

Dependent from their function, structure and substrate specificity, human caspases are subdivided into three groups. **Group-I** or inflammatory caspases (caspase-1, -4, -5, -12) are characterized by the presence of a caspase recruitment domain (CARD) at the N-terminus and become activated by initiator caspases (Thornberry, Rano et al. 1997). **Group-II**, or effector caspases, (caspase-3, -6 and -7) are responsible for the cleavage of cellular components during apoptosis. **Group-III**, or initiator caspases (caspases-2, -8, -9, -10) are characterized by the presence of protein-interacting domains in their N-terminus (Alenzi, Lotfy et al. 2010). Caspases are essential components during apoptosis. They function by cleaving cytoskeletal and nuclear proteins critical for maintenance of cell structure, and enzymes involved in metabolism and repair (Gorman, Orrenius et al. 1998). The caspases are synthesized as inactive pro-enzymes (zymogens) and, after appropriate apoptotic signaling, they are converted into mature enzymes by the formation of heterodimers that contain the active catalytic unit. Caspases with large prodomains are thought to be involved in the initiation of the apoptosis response and known as initiator caspases. Caspases with short prodomains are apparently activated by the initiator caspases and known as effector caspases (Fig. 8) (Nicholson and Thornberry 1997; Gupta 2000).

Caspase-3 is the major effector caspase, which cleaves many of cellular substrates during apoptosis (Porter and Janicke 1999). Caspase-7 is highly similar to caspase-3 and demonstrates similar substrate specificity (Fuentes-Prior and Salvesen 2004).

The initiator caspases (usually caspase-8) cleave Bid, the link between the death receptor and the mitochondrial apoptosis pathway and leads to Bax activation and cytochrome c release (Liu, Zou et al. 1997) (Li, Zhu et al. 1998; Steemans, Goossens et al. 1998).



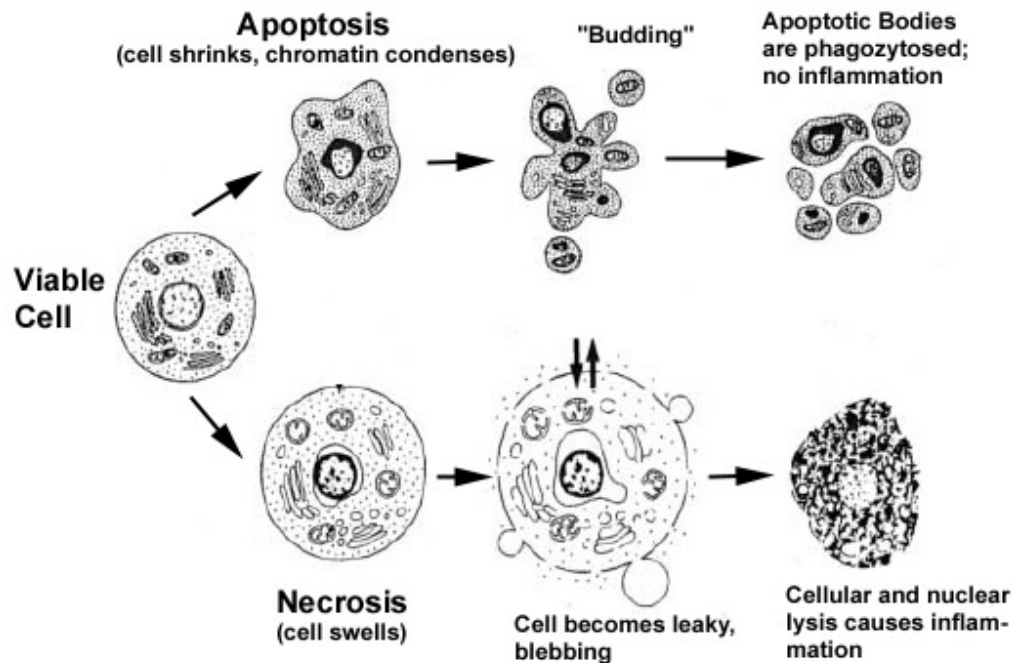
**Fig. 8 Caspase structure (Riedl and Shi 2004).** The caspase family. Three major groups of caspases are presented. Group I: apoptosis effector caspases, group II: apoptosis initiator caspases. The CARD, the DED, and the large (p20) and small (p10) catalytic subunits are indicated.

#### 1.4.4 Necrosis

Necrosis, is a non apoptotic form of cell death characterized by depletion of cellular ATP, plasma membrane rupture, cell swelling, and marked inflammation (Holler, Zaru et al. 2000; Xu, Tavernarakis et al. 2001; Baines, Kaiser et al. 2005; Nakagawa, Shimizu et al. 2005; Schinzel, Takeuchi et al. 2005). It has been reported that many cell types that can not initiate or propagate the apoptotic signaling cascade do not survive, but die by necrosis which is also a regulated process (Fiers, Beyaert et al. 1999; Festjens, Vanden Berghe et al. 2006).

Some pathophysiological processes, such as ischemia–reperfusion (I/R), inflammation, reactive oxygen species (ROS)-induced injury and glutamate excitotoxicity, induce necrotic cell death *in vivo* (Vanlangenakker, Vanden Berghe et al. 2008). Mitochondria-derived ROS are an absolute requirement in necrotic killing of L929 cells by TNF, and are also responsible for the ultrastructural changes in the mitochondria and endoplasmic reticulum (ER) during cell death (Schulze-Osthoff, Bakker et al. 1992). Necrotic cell death is represented in Fig. 9.





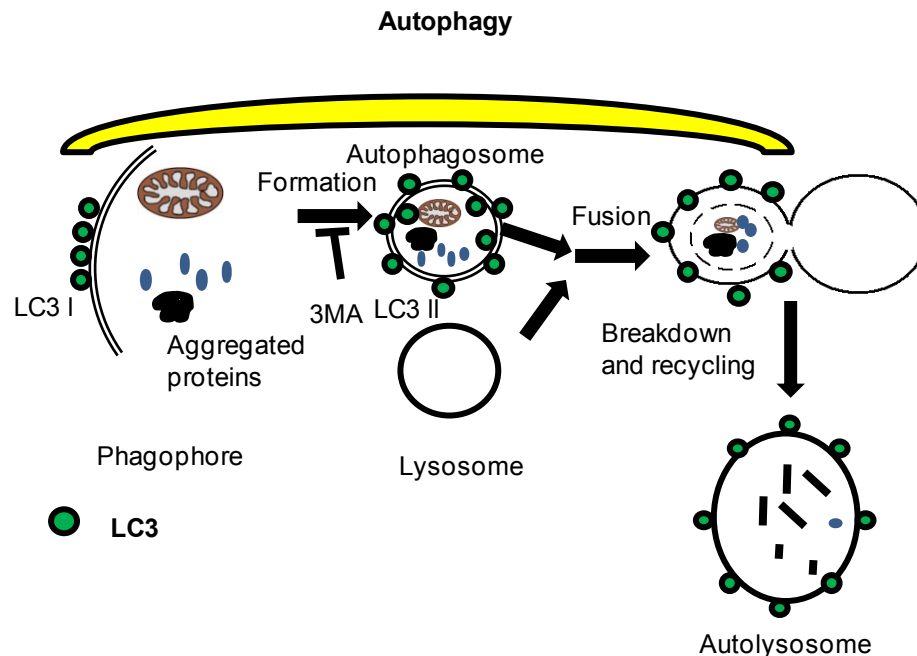
**Fig. 9 Hallmarks of the apoptotic and necrotic cell death process.** (Van Cruchten and Van Den Broeck 2002). While the process of apoptosis is characterized by cellular condensation, necrotic cells are swelling, during necrosis the nuclear structure is maintained until the membrane bursts due to the swelling and subsequently the intracellular components are released and may lead to acute inflammatory responses.

#### 1.4.5 Autophagy

Autophagy is a cellular catabolic degradation in response to cellular stress conditions or starvation of cells. During autophagy, organelles and cytoplasm are digested and recycled in order to maintain cellular metabolism and homeostasis (Levine and Klionsky 2004; Mizushima 2007). Autophagy is regulated by mTOR downstream of PI3 kinase/AKT, which regulates cell growth and protein synthesis. However, how exactly autophagy is regulated requires further investigations (Mathew, Karantza-Wadsworth et al. 2007).

Tumors resistant to apoptosis, need autophagy to survive metabolic stress (Degenhardt, Mathew et al. 2006; Karantza-Wadsworth, Patel et al. 2007; Karantza-Wadsworth and White 2007). On the other hand, autophagy has been considered also as type II programmed cell death (Edinger and Thompson 2003; Levine 2005). In addition it was reported, that treatment of apoptosis resistant cancer cells with cytotoxic drugs may induce caspase independent cell death by autophagy and that makes it important for further investigations (Shimizu, Kanaseki et al. 2004; Boya, Gonzalez-Polo et al. 2005; Colell, Ricci et al. 2007; Karantza-Wadsworth, Patel et al. 2007).

An autophagosome marker is the protein LC3 which is cleaved during autophagy. LC3 cleavage involves conversion of the LC3-I isoform into LC3-II that correlates with the autophagosome formation. LC3-II is the only protein known that specifically associates with autophagosomes (Kabeya, Mizushima et al. 2000) (Fig. 10).

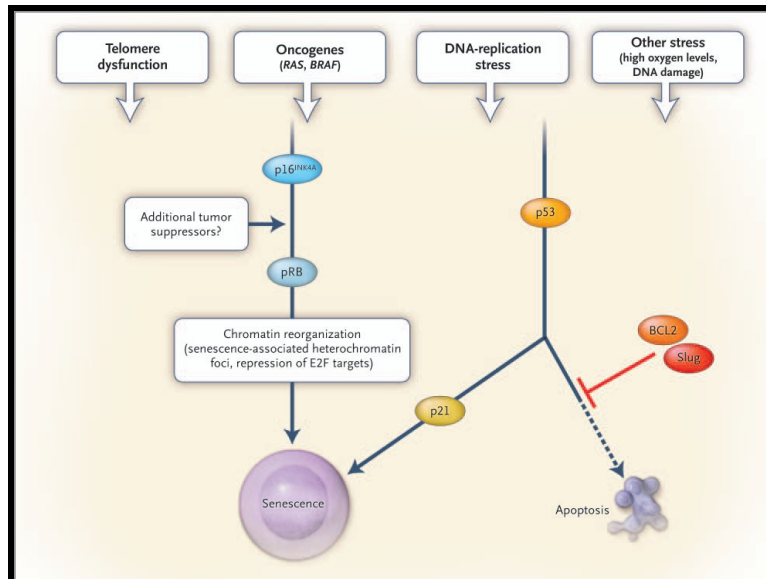


**Fig. 10 Autophagy pathway.** The distinct stages are controlled by atg gene products. The vesicle nucleation and vesicle elongation process in the initial stages of autophagy sequester the cytosolic contents and result in the formation of the autophagosome, a double-membrane vesicle. The outer membrane then fuses with the lysosomes to form the autolysosome and the internal content is degraded by lysosomal hydrolases.

#### 1.4.6 Senescence

Cells which stop dividing become senescent and lose the ability to proliferate (Pani ; Chang, Broude et al. 1999; Itahana, Campisi et al. 2007; Rodier, Coppe et al. 2009) The phenotype of senescent cells is characterized by large or flat morphology. Cellular senescence is a metabolically active form of irreversible growth arrest and it is regulated by tumor suppressor pathways like p53 and p16 INK4A/pRb (Fig.11). This complicated cellular event is initiated in response to extracellular or intracellular stress (Davalos, Coppe et al. ; Campisi 2001; Wright and Shay 2001; Ben-Porath and Weinberg 2005) (Kulju and Lehman 1995; Narita, Nunez et al. 2003). This observation leads to the hypothesis that senescence may be used as a tumor suppressor mechanism (Collins and Sedivy 2003; Ohtani, Yamakoshi et al. 2004; Gil and Peters 2006).

Normal cells can also undergo replicative senescence which is associated with a shortening of the telomeres after each cycle of DNA replication (Harley, Futcher et al. 1990; Vaziri and Benchimol 1998).



**Fig. 11 Cellular senescence (adapted from (Mooi and Peeper 2006)).** Senescent cells have been observed in normal aging and in cells/tissues of various age related pathologies. It is regulated by tumor suppressor pathways like p53 and p16 INK4A/pRb.

## 1.5 Radiation therapy

Ionizing radiation induces damage to cellular proteins, lipids and nucleic acid. The key consequence of ionizing radiation is DNA lesions from which DNA double strand breaks (DSBs) have a pivotal role in the decision whether the cell would survive the radiation exposure (Hall and Giaccia 2006). The response to DNA damage could lead to activation of DNA repair pathways or cell cycle arrest and cell death via apoptosis (Jackson 2001).

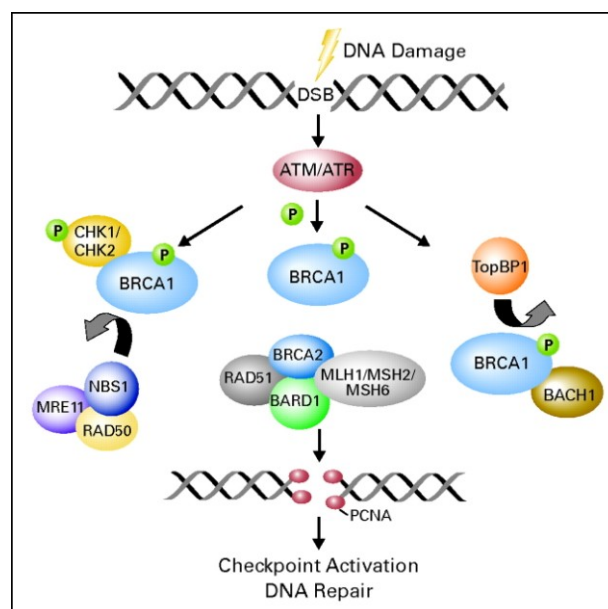
Exposure to ionizing radiation results in the immediate production of free radicals that last few milliseconds. In presence of O<sub>2</sub>, free radicals induce intracellular oxidative stress by other reactive oxygen species including hydrogen peroxides (H<sub>2</sub>O<sub>2</sub>) and organic superoxides (ROOH). These processes induce redox sensitive signaling pathways that are suggested to induce cytotoxicity, cell cycle delay and genomic instability (Spitz, Azzam et al. 2004).

As it was mentioned in paragraph 1.2, tumor hypoxia is a cause of radioresistance due to lack of O<sub>2</sub>, as oxygen is required to make permanent

and irreparable the DNA damage by ionizing radiation in the radiochemical process (Thomlinson and Gray 1955; Overgaard 2007)

### 1.5.1 DNA double strand breaks (DSBs)

Major aim of classical chemo- and radiotherapy in anticancer treatment is the eradication of clonogenic tumor cells. The aim of ionizing radiation is to induce cellular stress and DNA damage by interacting either directly with the DNA or via the formation of reactive oxygen species (ROS) which can subsequently damage cellular structures and macromolecules. DNA double-strand breaks belong to the most serious lesions and are likely to be recognized by either of the kinases ATM, ATR or DNA-PKc which represent members of the phosphatidylinositol-3-OH-kinase-like family of protein kinases (PIKKs) (Fig. 12). ATM and ATR are responsible for the phosphorylation of the core histone protein H2AX. Phosphorylated H2AX, is a potent coordinator of DNA-repair mechanisms (Kinner, Wu et al. 2008). In case that the DNA damage is too intense, the cell will undergo p53-induced apoptosis or cell cycle arrest (Roos and Kaina 2006)



**Fig. 12 DNA damage response.** (Weberpals, Clark-Knowles et al. 2008)

## 1.6 The aim of the study

Aim of the current study was to propose a novel strategy to overcome therapy resistance of tumor cells under acute hypoxia by using the radical-forming endoperoxide Dihydroartemisinin (DHA).

First, it was important to focus on the efficacy of DHA on solid tumors under normoxic and hypoxic conditions. To answer this question, colon cancer cells were chosen (HCT116, colo205 and HCT15) for *in vitro* experiments.

The next question applied was to elucidate which death pathway was induced in solid tumors such as colon cancer and prostate cancer and in Jurkat T-lymphoma cells, in response to DHA, focusing on apoptosis, necrosis and autophagy death modes.

Moreover, considering the importance of the pro-apoptotic proteins Bax and Bak, details of the mitochondrial apoptotic mechanism in response to DHA treatment under normoxia and hypoxia were further investigated using colon cancer cells, which were either Bax/Bak positive or Bax or/and Bak negative. As DHA is a ROS-donor, it was important to evaluate its antineoplastic efficacy, focusing on the role of hypoxia for its cytotoxic effects and its potentiality in combination therapy with ionizing radiation. For this purpose, HCT116 wt cells treated with DHA under normoxic and hypoxic conditions were used. Both short-term and long-term experiments were performed in order to understand the involved mechanism which was activated in case of the single treatments as well as in combination treatments with radiotherapy

## 2 Material & Methods

### 2.1 Materials

**Table 1: Materials**

Name	Company
2-propanol (isopropanol)	Sigma
$\beta$ -mercaptoethanol	Calbiochem
Acrylamide- / bisacrylamide (Gel 30) solution	Roth
Ammoniumperoxodisulphate (APS)	Roth
Bovine serum albumine (BSA)	New England Biolabs
CCCP	Calbiochemy
Citric acid	Roth
Coomassie Brilliant Blue	Fermentas
Dihydroartemisinin (DHA)	Sigma
Dimethylsulphoxide (DMSO)	Sigma
Phosphate Buffered Saline (PBS)	Gibco
DTT (1,4-dithiothreitol)	Sigma
Ethanol (EtOH)	Merck
Fetal Bovine Serum (FBS)	Biochrom
Formaldehyd	Merck
Glycerol	Merck
Glycine	Roth
Guanidinium hydrochlorid	Roth
Hoechst 33342	Sigma
HCl	Roth
KCl	Merck

Name	Company
MgCl <sub>2</sub>	Merck
Methanol	Merck
Milk powder	Roth
Mitotracker Red	Invitrogen
Moviol	Calbiochem
Natrium-Citrat	Sigma
NaCl	Merck
NaOH	Roth
Paraformaldehyde (PFA)	Merck
Ponceau-S	Fluka
Phalloidin FITC TRIZ	Sigma
Propidium iodide (PI)	Roth
Protein marker for SDS page	Fermentas SM1811
Potassium Ferrocyanide	Sigma
Potassium ferricyanide	Sigma
RPMI 1640	PAA
X-gal	Roth
Sodium dodecyl sulphate (SDS)	Roth
TMRE	Molecular probes
Tris-base	Roth
Tris-HCl	Fluka
Triton X-100	Sigma
Trypan blue	Sigma

Name	Company
Trypsine (0.05 % in EDTA)	PAA
Tween 20	Sigma
TEMED (N,N,N',N'-Tetramethylethyldiamin)	Roth
Wst – 1	Roche

### 2.1.2 Antibodies

**Table 2: Antibodies**

Name of primary antibody	Company
Anti- $\beta$ -aktin (Mouse)	Sigma
Anti-Bax (rabbit)	Cell Signaling
Anti-Bcl-xl (rabbit)	Cell Signaling
Anti-Becclin (rabbit)	Cell Signaling
Anti-PARP (rabbit)	Cell Signaling
anti-cleaved PARP(rabbit)	Cell Signaling
Anti-LC3 B (rabbit)	Cell Signaling
Anti CD71 (mouse)	Santa Cruz
Anti AIF (rabbit)	Cell Signaling
Anti-Mcl-1 (Mouse)	Cell Signaling
Anti-HIF-1 $\alpha$ (Mouse)	BD Biosciences
Anti-P-H2AX (rabbit)	Cell Signaling
Anti-cytochrome-c (rabbit)	Pharmingen
Anti-caspase-3 (rabbit)	Cell signaling
Anti-cleaved Caspase-3 (rabbit)	Cell signaling



Name of primary antibody	Company
Anti-Noxa (Mouse)	Calbiochem
Annexin-V FITC ( for FACS)	BD Biosciences
Anti-Puma (rabbit)	Cell Signaling
Anti-Tubulin (rabbit)	Cell Signaling

Name of secondary antibody	Company
Anti-rabbit-IgG Cy2	Amersham
Anti-rabbit-IgG HRP (goat)	Amersham
anti-mouse HRP	Amersham
Anti-rabbit HRP	Amersham

### 2.1.3 Technical equipment

**Table 3: Technical equipment**

Name	Company
Elisa Reader	Synergy HT Bio-Tek
Inverted light microscope	Leica DMIRB
Fluorescence microscope	Leica (090-131.001)
Confocal-Laser- fluorescence microscope	Leica TCS SP 5
Hypoxia Chamber	Invivo <sub>2</sub> 400, RUSKINN
Microscope Camera	Zeiss Axiocam
Steril Bench	BDK
Western Blot Developer	Agfa Curix 60
Centrifuge	Jouan CR422
Apotome	Zeiss Axiocam

Name	Company
FACS Calibur Flow Cytometer	BD Becton Dickinson
GelCount™	Oxford optronix
Blotting chamber	Bio Rad
Power supply	Bio Rad
Polyacrilamid gel documantation system	Bio Rad
Laboratory balance	KERN
Bench centrifuge	Eppendorf
Gilson pipets	Eppendorf
Cell culture incubator	Thermo Sientific
Watter bath	Köttermann
Vortex mixer	Bender & Hobein

#### 2.1.4 Other materials

**Table 4: Other materials**

Name	Company
Pipetboy	Eppendorf
PVDF membrane	Roth
Films	Thermo Sientific
Plastic pipets (5-50ml)	Sarstedt
Plastic cell culture plates	Corning Incorporated
Whatman filter paper	Schleicher & Schuell
Cell culture flasks (75 / 175 cm <sup>2</sup> )	Sarstedt
Microscope slide	Engelbrech
Glass coverslips	Roth

Name	Company
Eppendorf tubes (1.5-2.5ml)	Sarstedt
FACS tubes	BD Falcon

### 2.1.5 Software

Image J

Photoshop

Graph Pad prism

KC4

ImageQuant 5.2 (GE-Healthcare)

AxioVision Rel. 4.5

### 2.1.6 Buffers and Reagents

**Table 5: Buffer for Immunofluorescence**

<b>PBS</b>	1x
<b>PFA</b>	2% in PBS
<b>Triton X-100</b>	0,2% in PBS
<b>BSA</b>	1% in PBS

**Table 6: Buffer for SDS page and Western Blotting**

<b>CST lysis buffer</b>	62.5mM Tris-HCl (pH 6.8) 2% (w/v) SDS 10% (w/v) glycerol 50mM DTT 0.01% (w/v) bromophenolblue
<b>Stacking gel buffer</b>	60.6g/l Tris-base 0.4% (w/v) SDS pH 6.8
<b>Running gel buffer</b>	18.17g/l Tris-base 0.4% (w/v) SDS pH 8.8
<b>APS</b>	10% ammoniumpersulphate in H <sub>2</sub> O
<b>TBS</b>	150mM NaCl 10mM Tris-base

	dH <sub>2</sub> O pH 7.6
<b>TBS-Tween</b>	TBS + 0.05% (v/v) Tween-20
<b>Blocking buffer (Western Blotting)</b>	5% milk powder in TBS
<b>Transfer buffer (10x)</b>	30,3g Tris Mw=121,1 144g glycine Mw=75,07 1l H <sub>2</sub> O
<b>Transfer buffer (1x) with methanol</b>	500ml 10x blotting buffer 1l MetOH 3.5l dH <sub>2</sub> O
<b>Stripping buffer</b>	62.5mM Tris-base pH 6.2 2% SDS 100mM $\beta$ -mercaptoethanol

Table 7: Buffer for polyacrylamide gel preparation

	<b>6% (stacking gel)</b>	<b>12% (running gel)</b>	<b>15% (running gel)</b>
<b>H<sub>2</sub>O</b>	5.5 ml	13.2 ml	9.2 ml
<b>Gel 30</b>	1.3 ml	16.0 ml	20.0 ml
<b>Running gel buffer</b>	-----	10.0 ml	10.0 ml
<b>Stacking gel buffer</b>	1.0 ml	-----	-----
<b>20% SDS</b>	80 $\mu$ l	400 $\mu$ l	400 $\mu$ l
<b>10% APS</b>	80 $\mu$ l	400 $\mu$ l	400 $\mu$ l
<b>TEMED</b>	8 $\mu$ l	16 $\mu$ l	16 $\mu$ l

Table 8: Buffer for FACS staining

<b>Nicoletti Staining Solution (Propidium iodide)</b>	0.1 % sodium citrate 0.1 % Triton X-100 50 $\mu$ g/ml propidium iodide
<b>TMRE</b>	25 nM TMRE in RPMI medium

**Table 9: Buffer for X-Gal staining solution**

<b>X-gal</b>	1 mg/ml
<b>Potassium ferrocyanide</b>	5 mM
<b>Potassium ferricyanide</b>	5 mM
<b>NaCl</b>	150 mM
<b>MgCl<sub>2</sub></b>	2 mM
<b>Citric acid</b>	40 mM, pH 6.0

### **2.1.7 Stock preparation**

DHA was dissolved to the final concentration of 40 mM stock in absolute EtOH. zVADfmk was dissolved in DMSO in final concentration of 20 mM stock

## 2.2 Methods

### 2.2.1 Cell culture

Colon cancer cell lines (HCT116, Colo 205 and HCT15) were cultured with RPMI medium plus 10% FCS (Fetal Calf Serum). In case of the HCT116 cell line, other clones were used which were wt or deficient for either Bax or Bak, or both negative. Those cells were kindly provided by Professor Dr. Peter Daniel (Zhang, Yu et al. 2000). Jurkat T-Leukemia cells were cultured in RPMI medium plus 10% FCS. The Du145 prostate cancer cell line was also kindly provided by Professor Dr. Peter Daniel. Du145 cells used for the experiments were either DU145 mock (Bax negative/ Bak positive) or Du145 Bax (which were reexpressing also Bax). The cells are listed in table 1

**Table 10: List of the cell lines**

Name	Characteristics	Medium
HCT116 wt	Bax/Bak positive, K-RAS mutated p53 wt	RPMI 10% FCS
HCT116 Bax negative	Bax negative / Bak positive	RPMI 10% FCS
HCT116 Bak negative	Bax positive /Bak negative	RPMI 10% FCS
HCT116 Duo Bax/Bak negative	Bax /Bak negative	RPMI 10% FCS
JCAM Bak positive	Bak positive/Bax negative	RPMI 10% FCS
Du 145 mock	Bak positive/Bax negative	RPMI 10% FCS
Du 145 Bax	Bax positive/Bak positive	RPMI 10% FCS
Jurkat A3	FADD and caspase-8 positive	RPMI 10% FCS
Jurkat FADD negative	FADD negative	RPMI 10% FCS
Jurkat caspase 8 negative	Caspase 8 negative	RPMI 10% FCS
HCT15	P53 mutated	RPMI 10% FCS
Colo205	P53 wt	RPMI 10% FCS

### 2.2.2 Passaging of the cells

Jurkat T-Lymphoma cells were cultured in 75 cm<sup>2</sup> flasks for suspension cells and passaged at the density higher than 70 %. Adherent cancer cell lines were cultured in 75 cm<sup>2</sup> flasks for adherent cells. To avoid clonal selections, the cells were passaged when 70 % confluence was reached and cultured for not more than 10 passages. In case of adherent cells, trypsin-EDTA was used for detaching the cells from the plastic. The cells were first washed with PBS in order to remove the FCS, which is inhibiting the trypsin. After the washing step, cells were incubated for 2-3 minutes with trypsin-EDTA at 37°C. Finally, cells were resuspended with RPMI with 10% FCS in order to inhibit trypsin. The cell suspensions were further diluted 1:10 in fresh medium. In case of Jurkat cells, the passaging was the same as for adherent cells, except of the trypsinisation step. Incubation of the cells was carried out at 37°C, 5% CO<sub>2</sub> and a pO<sub>2</sub> of 21%.

### 2.2.3 Counting of the cells

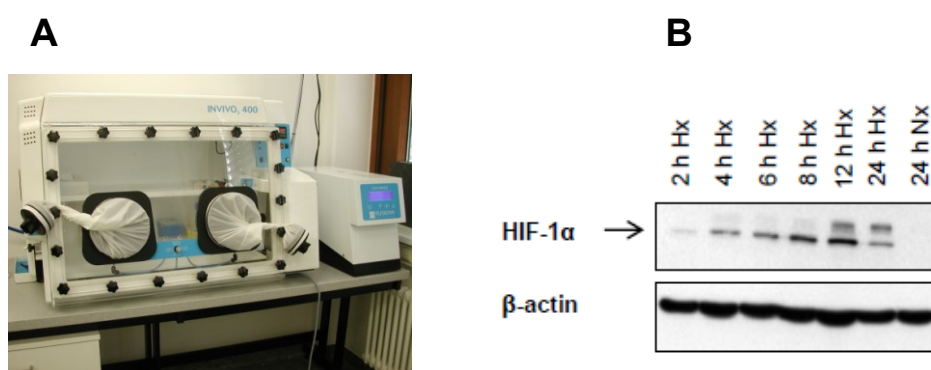
The cells were diluted 1:1 with trypan blue, placed into a Neubauer counting chamber and counted. Trypan blue is a dye that stains cells with damaged membranes and can be therefore used in order to distinguish living and dead cells. Only the number of living cells (shiny bright) was calculated via the following formula: cells/ml = (whole number of cells /4) x (dilution factor) x 10<sup>4</sup>.

### 2.2.4 Freezing the cells

The cells were expanded and frozen as follows. The cells were detached with trypsin - EDTA and centrifuged in 1000 rpm for 4 minutes at 4°C. The pellet was resuspended in freezing medium containing 20% FCS and 10% DMSO. Every step was performed on ice (DMSO prevents membrane damage during freezing, but could be toxic for the cells at room temperature). The vials were finally placed overnight at -80°C in an isopropanol-filled cooling box and the day after transferred into liquid nitrogen. Cells were reactivated at any time for the future laboratory needs.

### 2.2.5 Cell treatment

For each experiment, the appropriate amount of cells was plated in order to have 70 % confluence 24 hours later. At this time, cells were treated. Hypoxia treated cells were additionally pre-incubated for 2 hours at 0.2 %  $pO_2$  in an hypoxia chamber (Fig. 13) in order to allow the cells to adapt to those conditions. DHA concentrations applied were in the range of 0-80  $\mu M$ . To study caspase dependency of the observed effects, some samples were treated with 20  $\mu M$  pancaspase inhibitor ZVAD fmK 30 minutes before DHA. In case of combination treatments with irradiation, the drug treatment was performed 10 minutes before the irradiation. Cells were irradiated with an Isovolt-320 X-ray tube (Seifert-Pantak) running at 320 kV, 10 mA, with a 1.65 mm aluminum filter. Irradiation was carried out at room temperature at a distance of 75 cm. The effective photon energy was  $\sim 90$  keV, the dose rate  $\sim 2.76$  Gy/min. The irradiation dose applied was in a range of 0-10 Gy.



**Fig. 13 (A)** Hypoxia chamber INVIVO<sub>2</sub> 400 **(B)** HIF-1α protein expression was used as a marker for hypoxia. Nx=Normoxia, Hx=Hypoxia.

### 2.2.6 Preparation of cell lysates for SDS – Page

For whole cell lysates,  $0.5 \times 10^6$  cells were plated in 10 cm Petri dishes and 24 h later treated with DHA (0-40  $\mu M$ ). After appropriate time points, supernatants and cells were collected in the same falcon tube and centrifuged 4 minutes at 1000 rpm, 4° C. The pellets were resuspended in 1ml of cold PBS, transferred to small eppendorf tubes (1.5 ml) and once more centrifuged at 2000 rpm for 3 minutes (4° C). Finally, the pellets were resuspended in CST buffer ( $10^6$  cells/100  $\mu l$  buffer) and the samples were denaturated for 10 minutes at 99° C in hot block. The cell lysates were stored at -20° C until use.



### 2.2.7 SDS – Page and western blotting

The cell lysates were loaded on 12 or 15 % polyacrylamide gels and SDS – PAGE was performed under denaturing conditions. In order to determine the molecular weight, the appropriate protein marker was used. The gels were set under an electrical charge of 80 V until the protein front line had reached the end of the stacking gel. Voltage was then shifted at 100 V for the remaining time.

The proteins were transferred to a PVDF membrane, previously incubated for few minutes in methanol, using the sandwich Western Blotting. The Western Blotting transfer chamber was filled with cold 20 % methanol blotting buffer for better results the transfer was performed over night at 100 mA at 4° C.

Following the transfer, to avoid unspecific binding of the antibodies, the membranes were blocked in 5 % milk in TBS at room temperature on a shaker. Subsequently, the membranes were usually incubated at 4° C over night with the primary antibodies appropriately diluted in TBS 5 % BSA or dried milk. The membranes were then washed three times with TBS-Tween (0.5 %) and incubated for 1 hour at room temperature with the secondary antibodies diluted 1:2000 in 5 % milk/TBS. Following the washing steps with TBS-Tween, detection of the desired protein bands was conducted with the application of the “Enhanced-chemiluminescence (ECL) Western Blotting Detection System”.

### 2.2.8 Evaluation of Western Blots by densitometric analyses

A densitometric analysis of Western blots was performed by Photoshop software as follows. After selection of the desired bands using the Marquee Tool, the analyses could be done by recording the grey value mean measurement. The same procedure was used for all the bands of interest and for the background, which was then subtracted from all the measurements. Finally, the quantifications were normalized dividing the obtained values with the values of an internal control, such as  $\beta$ -actin, set as 1 or 100 and expressed in the same amount in all the cells.

## 2.2.9 Fluorescence microscopy

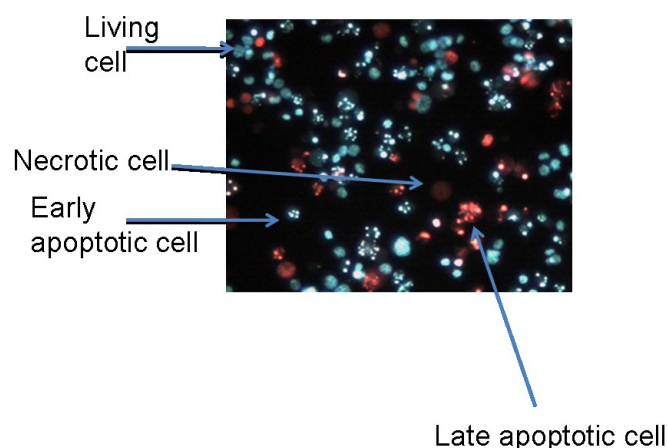
### 2.2.9.1 Immunofluorescence

Cells were placed on coverslips in multi-well plates and treated the following day. After appropriate time points, the plates were used for immunofluorescence. At first, the wells were washed three times with PBS and the cells were then fixed with 3 % PFA/PBS for 15 minutes at room temperature. After removal of the fixation buffer, the cells were permeabilized for 5-10 minutes with 0.2 % Triton X-100 in PBS. After 3 washing steps with PBS, unspecific binding sites were blocked with 1 % BSA in PBS for 30 minutes. The blocking buffer was removed and the cells were subsequently incubated  $\geq 1$  hour in a humid chamber at room temperature with the respective primary antibodies solution (usually diluted 1:100). After three washing steps with PBS (for 2 min), cells were incubated for 45 minutes incubation with secondary antibodies solution in the dark. Finally, for nuclei visualization, 1.5  $\mu$ M Hoechst 33342 in PBS was added to the cells and incubated for 10 additional minutes in the dark. After a final washing step, the coverslips were placed inversely onto fluoromount-G mounting medium or mowiol on microscope glass slides. The slides were dried and stored at 4° C. The analysis of the samples was conducted using the Leica TCS SP5 confocal laser scanning microscope. The pictures were taken by Zeiss camera using 63X oil objective. Cytochrome-c and LC3 proteins were labeled by cy2-conjugated secondary antibody which gives green color and can be detected in GFP filter. In case of  $\gamma$ -H2AX protein, cy3-conjugated secondary antibody was used, which gives red color and can be detected with rhodamine filter set. Finally Hoechst 33342 could be detected with the use of Dapi channel.

### 2.2.9.2 Staining of apoptotic and necrotic cells with Hoechst 33342 and PI

To discriminate the type of cell death, Hoechst 33342 and PI staining was performed. Hoechst 33342 is a fluorescent dye used for DNA-labelling in fluorescence microscopy. This lipophilic dye stains both living and dead cells even after fixation. Due to its ability to pass the membranes, it is routinely used to label intact or early apoptotic nuclei. Hoechst 33342 is excited by ultraviolet light at around 380 nm and emits blue fluorescent light at around 461 nm emissions maximum. Propidium iodide, instead, binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA (Suzuki, Fujikura et al. 1997). It is used as a DNA stain for

both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis, and microscopy, to visualize the nucleus and other DNA containing organelles (Lecoeur 2002). In contrast to Hoechst 33342, PI is membrane impermeable and generally excluded from viable cells and therefore, it is used as a marker for dead cells. The discrimination between necrotic, apoptotic and viable nuclei is shown in Fig. 14.



**Fig. 14 Staining for apoptotic and necrotic cells by Hoechst PI staining.** The basic principle of this technique is that intact nuclei or early apoptotic nuclei are stained only by Hoechst (homogenous blue or fragmented blue respectively), and PI can stain the nuclei only in case of necrosis or late apoptosis (homogenous red or fragmented red appearance).

For the fluorescence microscopy experiment with Hoechst 33342 /PI staining, cells were plated in 12-well plates. The cells were treated the day after plating in normoxia or hypoxia. At the appropriate time points, a 1:1 Hoechst 33342 and PI mixture was diluted 1:1000 directly in the cell supernatants and subsequently incubated in the dark for 10 minutes. Following the incubation time it was possible to visualize the nuclei in the fluorescence microscope using Dapi filter.

### 2.2.9.3 Mitotracker staining

MitoTracker Red is a red-fluorescent dye that stains mitochondria in living cells and its accumulation is membrane potential-dependent.

After plating, cells were treated as previously described. Mitotracker solution was used at a final dilution of 100 nM in fresh medium, from a stock solution of 1 mM in DMSO. After 30 minutes of incubation at 37 ° C in dark, the plates were washed three times 2 minutes with PBS and the cells were fixed with 3 % PFA/PBS and counterstained (e.g. Hoechst 33342) or directly analyzed by fluorescence microscopy

## **2.2.10 Flow cytometry**

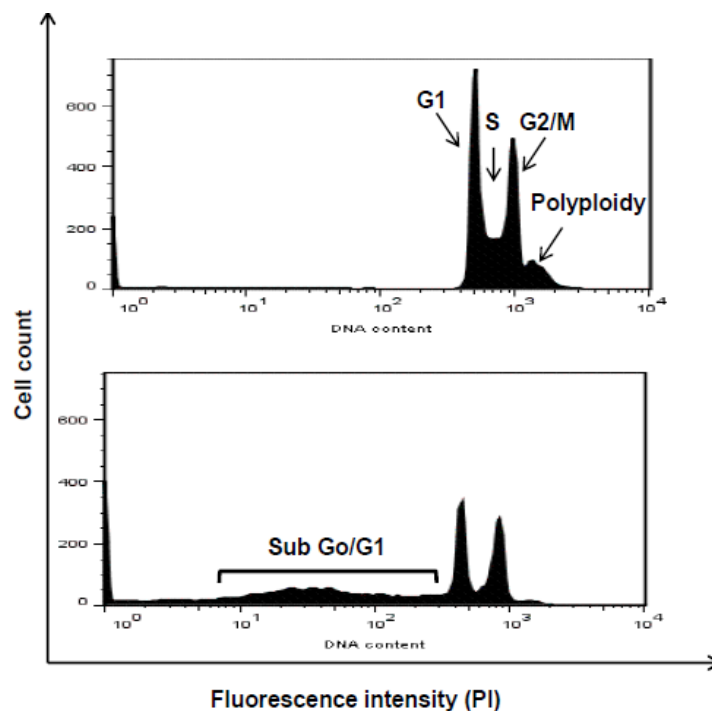
### **2.2.10.1 Principle**

Flow cytometry is a technique for counting and examining microscopic particles, e.g. cells, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second. Flow cytometry has many applications in both research and clinical practice. Common variation is to physically sort particles based on their properties, to purify populations of interest. Cells from a given cell suspension are accelerated by high pressure through a capillary thereby creating a regular stream of individual cells. The cells then pass an argon laser at 488 nm. The scattering of the incoming by the cells light which is detected by photo detectors can be divided into forward light scattering (FSC) and side scattering (SSC), which is scatter in a right angular compared to the incoming laser beam. While FSC provides a measure for the size of the cells, SSC is associated with cellular morphology and intracellular granularity. If cells are labelled with a fluorochrome-conjugated antibody or fluorescent dye before they are applied to the FACS machine, the incoming laser beam has to be adjusted according to the appropriate fluorochrome. The fluorochromes are then transferred to an excited state and start to emit light of a specific wavelength. By the use of band pass filters emissions of only a specific wavelength can be selected for detection. In the FACS Calibur machine (BD Transduction Labs) there are three different channels available, termed FL1, 2 and 3, which detect wavelengths of 537 nm, 597 nm and 650 nm, respectively. Typically, the measurements of cell size and granularity are visualized in a dot-plot, whereas fluorescence intensities are shown as histograms or dot plots. In the case of histograms, the y-axis reflects the numbers of cells and the x-axis the light intensity.

### **2.2.10.2 Propidium iodide or “Nicoletti” staining for apoptosis and cell cycle analyses**

The “Nicoletti” staining (Nicoletti, Migliorati et al. 1991) provides a FACS-based method to measure the cell cycle distribution of a cell population. The method is based on the DNA-intercalating dye propidium iodide. Although PI cannot by itself permeate membranes, this obstacle is circumvented by the addition of the detergent Triton X-100 in a hypotonic citrate buffer, a lipophilic compound that accumulates within the cellular and nuclear membranes and thereby

permeabilizing them for PI. In the histogram, healthy cells show a distinct pattern that is characterized by sharp peaks for diploid cells (G0/G1 phase) and for tetraploid cells (G2/M phase). During apoptosis, chromosomal DNA is fragmented into pieces of about 185 base pairs or multiples and for this reason, the amount of DNA in apoptotic cells is visualized as a characteristic sub-G1 peak (Fig 15).



**Fig. 15 Cell cycle distribution from FACS analyses.** Typical profiles of cells stained with PI in hypotonic buffer containing 0,1 % sodium citrate, 0.05 % Triton X-100 and 50  $\mu$ g/ml PI. DNA content is used to visualize cell cycle by FACS. The sub-G1 peak is attributed to apoptotic cells because of the DNA fragmentation

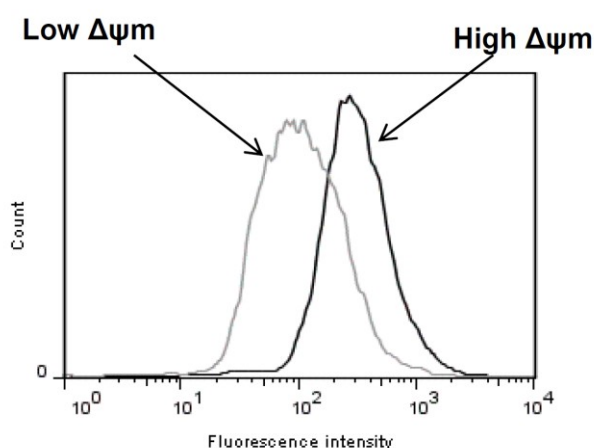
For this set of experiments, an appropriate number of cells was plated in 6-well plates and treated after 24 h. Cells were then harvested and centrifuged for 5 min at 1000 rpm at room temperature. The supernatant was discarded and the pellet resuspended in a hypotonic buffer containing 0.1% sodium citrate, 0.05% Triton X-100 and 50  $\mu$ g/ml propidium iodide. After one hour incubation at room temperature in the dark, the samples were measured in the fluorescence channel FI-2. Cellular debris has been excluded from the measurement by the appropriate gating settings during the evaluation.

### 2.2.10.3 Analysis of mitochondrial membrane potential with TMRE staining

The cationic, lipophilic dye tetramethylrhodaminethyl ester (TMRE) accumulates at the inner mitochondrial membrane in cells with an intact proton gradient and membrane potential ( $\Delta\psi_m$ ) (Scaduto and Grotyohann 1999).

TMRE has therefore been used in FACS analysis as a detector for living cells with intact mitochondria (FL-2 channel). During apoptosis, the  $\Delta\psi_m$  decreases or become even completely lost due to mitochondrial damage and TMRE can no longer accumulate in the cells. In the histogram, viable cells are characterized by a stable peak of high fluorescence intensity. Apoptotic cell populations show a decreased fluorescence signal derived from the TMRE, staining resulting in a shift to the left of the original peak.

The procedure was conducted by harvesting treated cells after the desired incubation time by centrifugation (5 min, 1000 rpm at room temperature). The supernatant was carefully discarded and the pellet resuspended in 25 nM TMRE in cell culture medium. The cell suspension was then incubated for 30 minutes at 37° C and subsequently measured in the FACS Calibur. 10  $\mu$ M carbonyl cyanide m-chlorophenylhydrazone (cccp) for complete depolarization of the mitochondria, was added 10 minutes before the sample measurement as a positive control for low  $\Delta\psi_m$  (Fig 16).



**Fig. 16 Cells stained with TMRE.** Typical profiles of cells stained with TMRE and analyzed by flow cytometry using FL-2 channel for detection. Cells with intact mitochondria (living cells) are characterized by high  $\Delta\psi_m$  (high fluorescence intensity) and apoptotic cells are characterized as cells with low  $\Delta\psi_m$  (low fluorescence intensity).

#### 2.2.10.4 Annexin V / PI

In apoptotic cells, the membrane phosphatidylserine (PS) is translocated from the inner to the outer plasma membrane, thereby exposing to the external cellular environment (BD biosciences 2008) also during the subsequent necrosis, which can be then distinguished using the PI staining. Annexin V is  $\text{Ca}^{2+}$  concentration dependent that has a high affinity for PS. Annexin V is routinely tested in flow cytometric analyses. Cells were incubated for 15 minutes in the dark with FITC-coupled annexin V and PI in binding buffer. At the end the cells were further diluted with binding buffer to reach  $1 \times 10^5$  cells, and measured within 1 hour (FL-1= annexin V and FL-2= propidium iodide; BD Transduction Labs).

#### 2.2.10.5 Activation of Bax

Activation of Bax and Bak is essential in mitochondrial apoptotic pathway. Bax activation involves a conformational change which allows its translocation to the mitochondrial membrane. To detect this activated form, a specific antibody binding to the N terminus of Bax was used (Nechushtan, Smith et al. 1999).

Cells were plated in 6-well plates and harvested at the appropriate time points after treatment. Cells were permeabilized for 30 minutes on ice with 0.1 % Triton X100/PBS, and after a washing step with PBS, incubated for 15 minutes at room temperature in the blocking solution (10 % FCS/PBS) and stained for 30 minutes on ice with the first antibody Bax Nt solution (1:1000 in 10 % FCS/PBS). The cells were then washed with 10 % FCS/PBS and incubated with the secondary anti rabbit conjugated cy2 antibody (1:1000 in 10 % FCS/PBS) for 30 minutes on ice and in the dark. Finally, the cells were washed and resuspended in 100  $\mu\text{l}$  PBS and measured by flow cytometry in FL-1 channel. Unstained cells, cells only with the primary or the secondary antibody and isotype control were used to validate the results.

#### 2.2.11 Colony formation assay

To investigate the long term effects of DHA treatment, such as the eradication of clonogenic tumor, colony formation assay was performed. This method is especially important in cancer research laboratories to determine the effect of drugs or radiation on proliferating tumor cells (Hoffman 1991).

Colonies were counted ( $\geq 50$  cells/colony) under the microscope (5-fold magnification). The surviving fraction of treated cultures was calculated with the following formula: survival fraction= colonies counted/cells seeded  $\times$  (PE/100).

Plating efficiency is given by the number of colonies counted divided by the number of cells seeded x 100. The survival curves were established by plotting the log of the surviving fraction versus the treatment dose.

Here I employed two principles of colony formation assay, the direct and delayed plating.

#### **2.2.11.1 Direct plating**

In the direct plating assay, 200 to 1600 cells/well were plated in 6-well plates. After 24 h in normoxia the cells were treated in normoxia or hypoxia and further incubated approximately 10 days until the colonies were formed. In case of hypoxia, the cells were incubated in hypoxia chamber only for 24 h after treatment and subsequently incubated in normoxia. The experiment was stopped according to the controls, in the way that the colonies were not confluent and was still possible to discriminate single colonies in the plates. At the end of the experiment, cells were fixed in 3.7 % formaldehyde and 70 % ethanol and stained with 0.05 % coomassie brilliant blue.

#### **2.2.11.2 Delayed plating**

In case of delayed plating the cells were plated and treated into the flasks and after 24 h the cells were trypsinized and plated again (200-16000 cells/well). The experiment was stopped after 10 days with the same procedure mentioned for the direct plating.

#### **2.2.12 WST-1 Assay**

The stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells (Berridge, Herst et al. 2005). Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture and the color of the cells supernatant was changed dependent from the density of the cells. The absorbance can be detected using a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance at 450 nm wavelength directly correlates to the number of viable cells.

The experiment was performed according to manufacture instructions of Roche applied Science.



### 2.2.13 Pulsed-field gel electrophoresis (PFGE)

Asymmetric field inversion gel electrophoresis (AFIGE) is a form of PFGE suitable for the separation of relatively large DNA-fragments (Stamato, Guerriero et al. 1993). The agarose cylinders with the lysed cells were loaded on 0.5 % agarose gels prepared with 0.5 µg/ml ethidium bromide. Gels were run in 0.5 x TBE for 40 h at 8°C. Cycles of 50 V (1.25 V/cm) for 900 s into the direction of DNA-migration alternated with 200 V (5.0 V/cm) for 75 s into the opposite direction. Subsequently, gels were scanned with a fluorescence scanner and the fractions of DNA released (FDR) from the cylinders into the lanes were quantified with ImageQuant 5.2 (GE-Healthcare). Dose response curves were plotted as FDR versus radiation dose. These curves were used to calculate the equivalent Gy dose ( $D_{eq}$ ) values for each FDR that was measured at a given repair time point and the repair kinetics were plotted as  $D_{eq}$  versus time.

### 2.2.14 DNA repair

Ten minutes prior to irradiation, cells were treated with 5 µM of DHA, as required by the experimental protocol.. After irradiation, cells were promptly returned to the incubator. At specific times thereafter, cells were collected, cooled down to 4° C, embedded in 3 x 5 mm low melt agarose cylinders at  $1 \times 10^5$  cells/cylinder and subsequently lysed. For cells that were used to obtain dose response curves, the cells were first embedded in agarose blocks, cooled down to 4° C, and then irradiated and lysed.

Lysis took place in lysis solution (100 mM EDTA, 50 mM NaCl, 10 mM Tris, 2 % N-lauroylsarcosine and 0.2 mg/ml protease) for 18 h at 50° C. After lysis, cylinders were washed in washing solution (100 mM EDTA, 50 mM NaCl, 10 mM TRIS) for 1 h at 37° C and then treated with washing buffer containing 0.1 mg/ml ribonuclease A at 37 °C for 1 h (PFGE and DNA repair experiments were performed in collaboration with Prof. G. Iliakis and Dr. M. Moscariello).

### 2.2.15 Senescence staining

Senescence staining detects β-galactosidase activity at pH 6 in cultured cells. β-galactosidase activity at pH 6 is present only in senescent cells and is not found in immortal proliferating cells (Dimri, Lee et al. 1995).

The cells were plated for senescence in 60 cm Petri dishes at  $2.7 \times 10^5$  per dish. Cells were treated with DHA under normoxic or hypoxic conditions and the plates were further incubated for the required time points. The cells were first washed two

times with PBS and subsequently fixed with 3 % of PFA/PBS for 5 minutes. The washing step was repeated after the fixation. At the end, the x-gal staining solution was placed on the cells and incubated for 16 h at 37° C without CO<sub>2</sub>. The cells were washed with H<sub>2</sub>O two times and observed under light microscopy. As a positive control, lung fibroblasts were used, since these cells become senescent in tissue culture when they reach confluence

#### **2.2.16 Statistical data analyses**

Experiments were performed at least three times and data were compared using a two-tailed unpaired t-test, with a *P* value ≤0.05 considered as significant, using a GraphPad InStat software (La Jolla, USA).

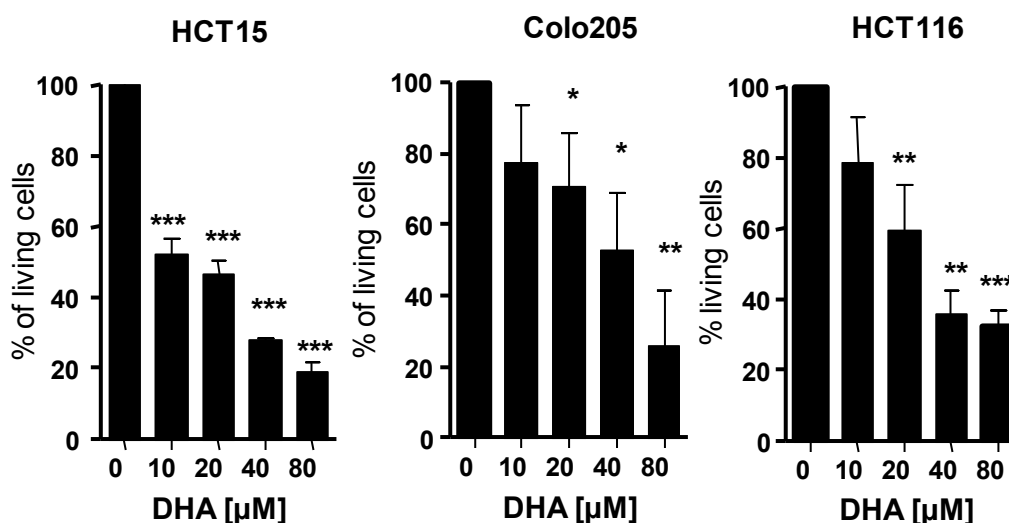
### 3 Results

#### 3.1 Antineoplastic efficacy of DHA

The first question was: whether DHA induced eradication of colon cancer cells in normoxia and hypoxia. The antineoplastic efficacy of DHA was studied in human colon cancer cells (HCT116, Colo205 and HCT115). For this purpose WST-1 assay (viability assay) was performed in those cells under normoxic and hypoxic conditions.

##### 3.1.1 DHA induces reduction of viability in colon cancer cells

Human colon cancer cell lines HCT116, Colo205 and HCT15 were treated with DHA (0-80  $\mu$ M) for 48 h. DHA efficacy was analyzed employing the WST-1 assay. DHA significantly reduced the number of viable cells in dose-dependent manner in all three tested cell lines compared to the untreated solvent control (EtOH) (Fig 17). The toxic concentrations of DHA ranged from 10-80  $\mu$ M DHA for HCT15 cells and 20-80  $\mu$ M DHA for Colo205 and HCT116. Significant differences regarding to cell viability were observed in all three tested cell lines. Cell viability reduced to 50 % in HCT15 cells in response to 10  $\mu$ M DHA treatment, reaching significant difference in comparison to its solvent control ( $p < 0.001$ ).



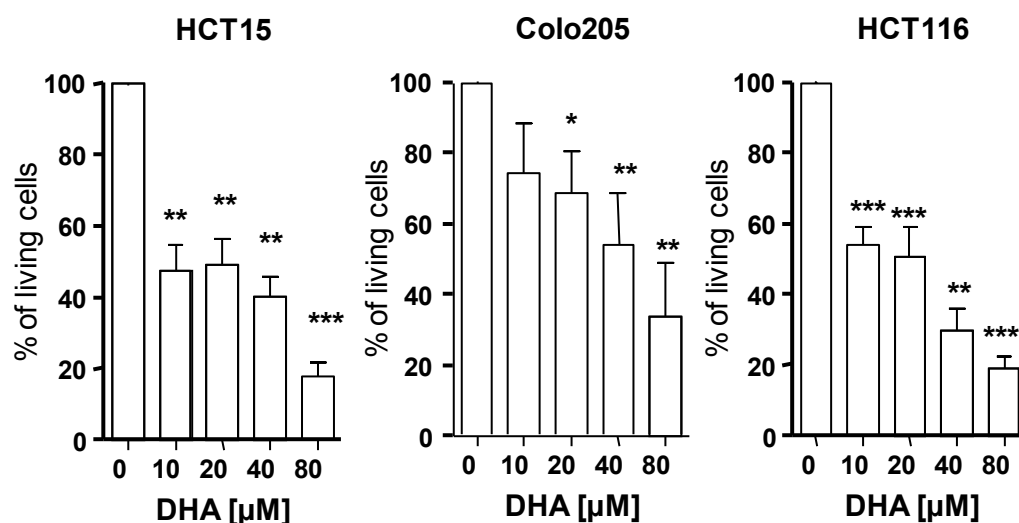
**Fig. 17 DHA induces reduction in the number of viable cells in normoxia.** HCT15, Colo205 and HCT116 cells were treated with increasing concentration of DHA. After 48h of incubation, WST-1 assay was performed. Ethanol was used as a solvent control of the drug. The experiments were performed in triplicate. The normalization was considered from ethanol controls. P values were calculated using unpaired two tailed t-test. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$

In addition, cell viability was further decreased in dose-dependent manner reaching 20 % viability in response to higher dose of DHA (80  $\mu$ M). In contrast to HCT15, the other two cell lines, Colo205 and HCT116, were more resistant to

DHA, with Colo205 to be the most resistant. However, cell viability was reduced in dose dependent manner reaching significant differences (20-80  $\mu$ M DHA).

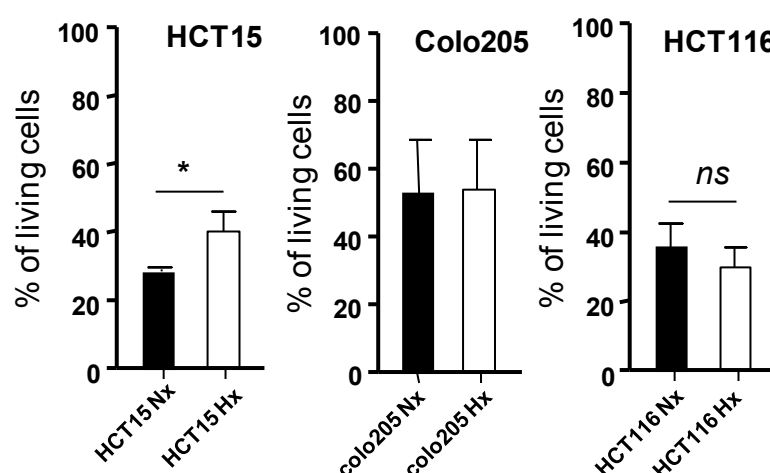
To gain insight into the putative drug activity, similar experiments were performed incubating the cells under hypoxia for 48 h.

The results showed similar antineoplastic activity of DHA under normoxic and hypoxic conditions (Fig.17-18). In contrast to normoxic conditions, in case of hypoxia in HCT15 cells, cell viability was significantly reduced at 10  $\mu$ M DHA but was not further decreased in dose dependent manner until the maximum dose of 80  $\mu$ M DHA. Colo205 cells showed similar response to the antineoplastic efficacy of DHA under both normoxic and hypoxic conditions. Finally, HCT116 cells were more sensitive to DHA treatment under hypoxic conditions than under normoxic conditions. Cells treated with lower DHA concentrations (10 and 20  $\mu$ M) showed similar reduction of cell viability (50%). In contrast to hypoxia, the same DHA concentrations in normoxia reached 80 and 60% of viable cells respectively. Higher DHA concentrations (40 and 80  $\mu$ M) induced strong reduction of viable cells under both normoxic and hypoxic conditions reaching significant difference ( $p < 0.001$ ) in comparison to solvent control. However, under hypoxic conditions DHA showed higher efficacy.



**Fig. 18 DHA is active under hypoxia and is cytotoxic in colon cancer cells.** Colon cancer cell lines were treated as described for normoxia experiment. Cells were incubated for 48 h in hypoxia and after were analyzed by WST-1 test. The experiment was performed at least in triplicate. The graphs represent the percentage of living cells evaluated from WST-1 test. The normalization was considered from ethanol controls. P values were calculated from unpaired two tailed t-test. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ .

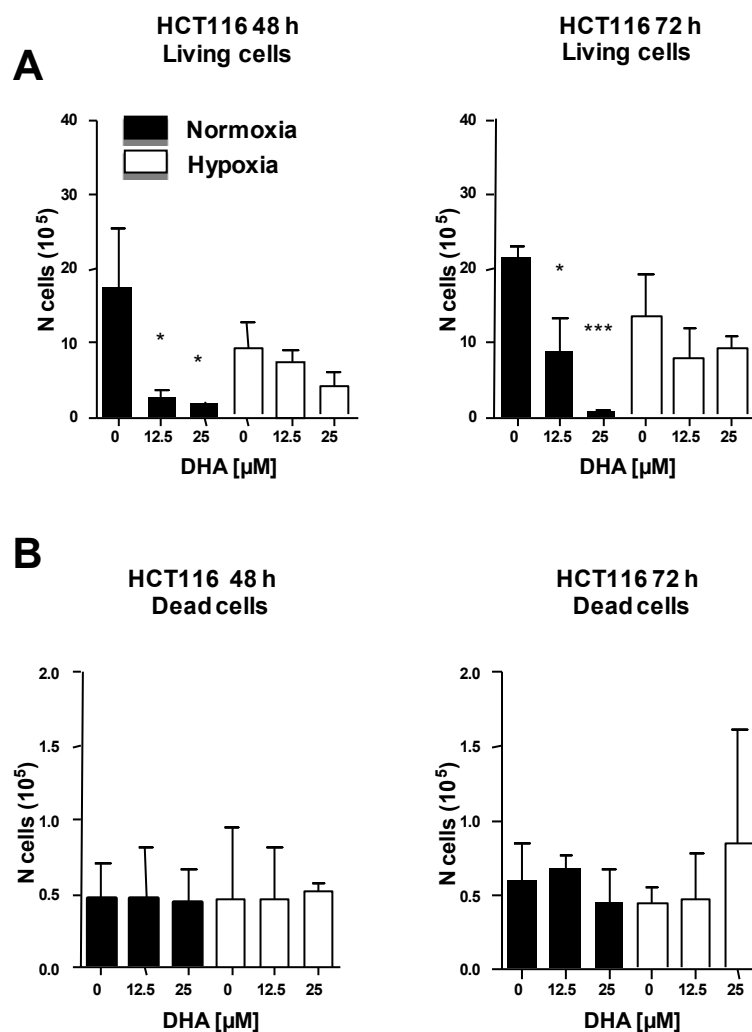
To compare the antineoplastic efficacy of DHA under normoxic and hypoxic conditions, cells treated with 40  $\mu$ M DHA from fig. 17-18 were further analyzed. As it was previously described, colo205 cells showed no differences between normoxia and hypoxia in response to DHA treatment. In case of HCT15 cells, DHA significantly reduced cell viability under normoxic conditions in comparison to the hypoxic conditions ( $P < 0.05$ ). Interestingly, HCT116 showed a higher sensitivity to DHA under hypoxic conditions; however, the reduction of viable cells did not reach significant difference (fig. 19).



**Fig. 19 DHA is active under both normoxic and hypoxic conditions and induces antineoplastic efficacy.** Data represent the percentage of living cells treated with 40  $\mu$ M DHA from the Fig.17 and 18. The normalization was considered from ethanol controls. Significant test was performed according to unpaired two tailed t-test. ns= not significant, (\*)  $P < 0.05$ . Hx=hypoxia and Nx=normoxia.

### 3.1.2 DHA reduces cell proliferation

WST-1 assay gives the information about the amount of viable cells. However, it does not give the information about the absolute amount of living/dead cells. To discriminate between the dead and viable cells and to determine whether DHA had a cytotoxic or a cytostatic effect, trypan blue staining was performed with HCT116 cells under normoxic and hypoxic conditions (Fig. 20).



**Fig. 20 DHA is cytostatic under normoxic and hypoxic conditions.** HCT116 cells were plated  $2 \times 10^5$ /well in 6 well plates and 24 h later were treated with DHA. At 48 h and 72 h the cells were harvested and stained with trypan blue. **(A)** The graphs represent the number of living cells in normoxia (black bars) and hypoxia (white bars). **(B)** The graphs represent the number of dead cells under normoxic (black bars) and hypoxic (white bars) conditions. The experiment was performed in triplicate. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$  unpaired two tailed t-test.

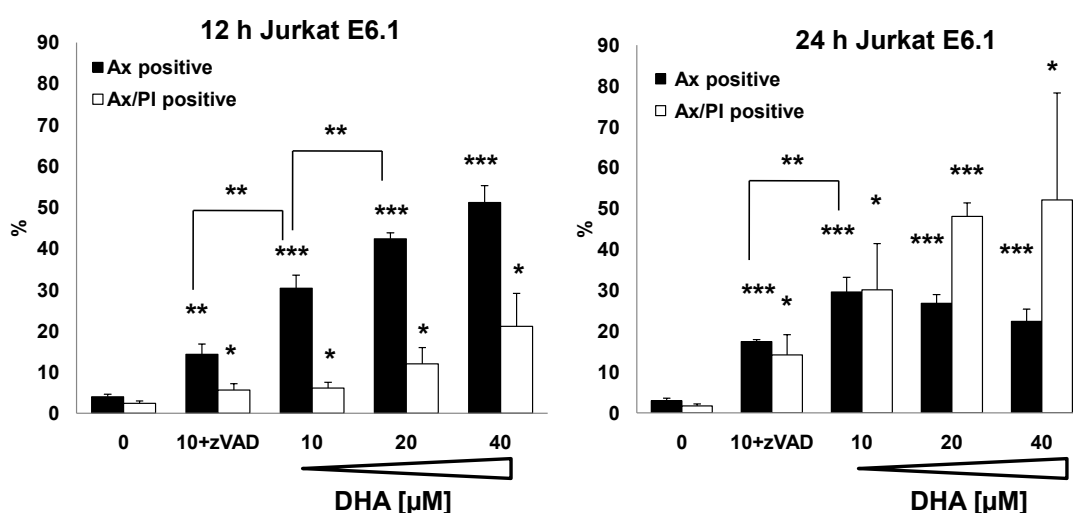
DHA had a cytostatic effect in both normoxic and hypoxic conditions. Especially, hypoxia alone could induce a cytostatic effect; however the difference was not significant. At lower drug concentrations the cells were able to grow after 72 h compared to 48 h, however the number of viable cells was significantly reduced in comparison to the control at both time points. At higher drug concentrations, cells were close to their plated number of cells  $2 \times 10^5$  in normoxia.

In case of hypoxia, proliferation inhibition exerted by DHA did not reach statistical significance in comparison to the ethanol controls. In addition, DHA did not increase the number of dead cells under both conditions at 48 h and 72 h time points. This indicated that DHA acts more as a cytostatic than as a cytotoxic agent.

### 3.1.3 DHA induces apoptosis and necrosis

Trypan blue staining gives the information about the living and the dead cells, however, it is not discriminating the apoptotic cells which still have intact membranes. To define whether DHA induced apoptosis or necrosis, tumor cells were further analyzed by fluorescence microscopy and flow cytometry.

Experiments with Jurkat E6.1 leukemic T cells were performed focusing on apoptosis induction. The cells were harvested 12 and 24 h after DHA treatment. For apoptosis detection, annexin V/PI staining was used as described before.



**Fig. 21 DHA –induced apoptosis is dose and time dependent.** Jurkat E6.1 cells were plated and 2 h later treated with DHA. After 12 and 24 h treatment the cells were harvested and analyzed by flow cytometry. Apoptotic cells were characterized the cells which were either only Annexin V positive (early apoptotic) only or annexin V plus PI positive (late apoptotic and necrotic). The experiment was performed in triplicate (Means  $\pm$  SD.;  $n = 3$ ; two-tailed  $t$ -test: (\*),  $P < 0.05$ ; (\*\*),  $P < 0.01$ ; (\*\*\*),  $P < 0.001$  (Handrick, Ontikatz et al. 2010)

Early apoptotic cells (annexin V positive only) and late apoptotic or necrotic cells (annexin V and PI positive) were quantified. DHA induced significant apoptosis and necrosis in dose and time dependent manner (Fig. 21). Apoptosis was strongly upregulated after DHA treatment already at 10  $\mu$ M DHA at 12 h time point (30 % of apoptotic cell) and further increased in dose dependent manner reaching significant difference between 10 and 20  $\mu$ M DHA ( $P < 0.01$ ). In parallel, cells in late apoptosis and necrosis (annexin V and PI positive cells) were also increased after treatment in dose dependent manner. At 24 h time point, apoptosis was upregulated in response to 10  $\mu$ M DHA (30 %) however, it did not increase further at higher dose of DHA (20 and 40  $\mu$ M), while late apoptosis and necrosis were significantly increased. At 12 h time point DHA induced maximum 20% of late apoptotic and necrotic cells, on the other hand at 24 h time point DHA induced 50

% of late apoptotic and necrotic cells indicating time dependency. These results indicate that DHA-induced apoptosis is an early event and it reaches the maximum at 12 h time point in response to 10-40  $\mu$ M DHA. At 24 h time point most of the cells are either late apoptotic or necrotic. Interestingly, the combination of annexin V positive with annexin V/PI positive cells, reaches 90 % at 24 h time point in response to 40  $\mu$ M DHA.

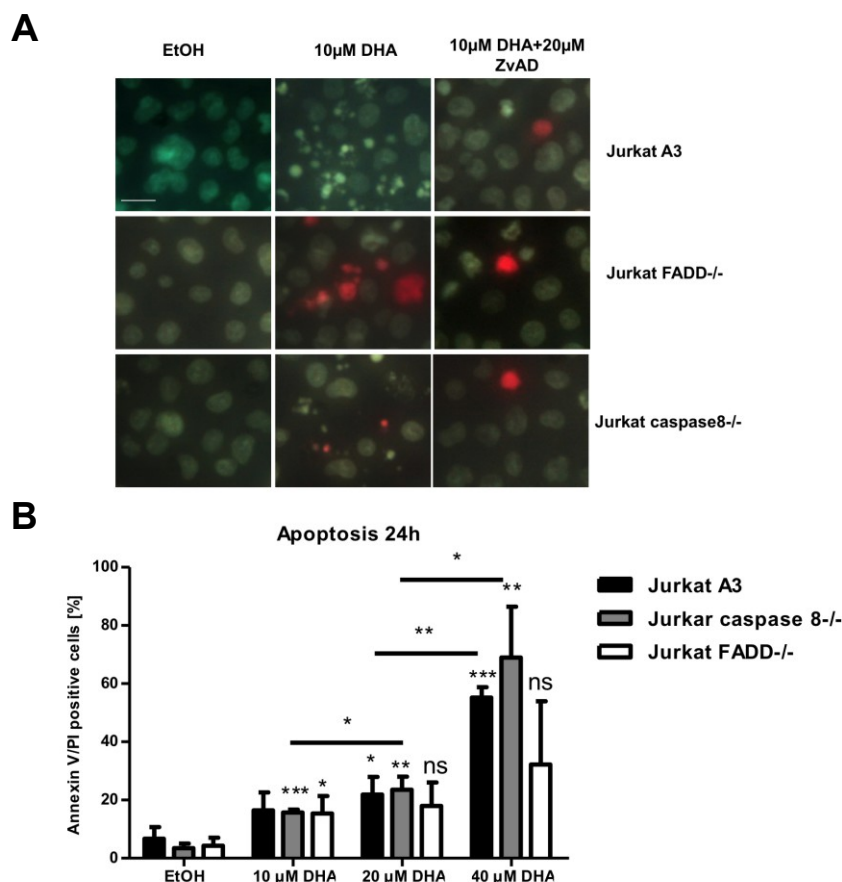
#### **3.1.4 DHA induces apoptosis via intrinsinsic pathway**

To further investigate the mechanism of antineoplastic action of DHA and to gain insight into the involved pathway, Jurkat A3 cells, or either FADD-negative or caspase-8 negative were treated with DHA and examined for apoptosis induction. FADD and caspase-8 are the key regulators in the extrinsic apoptosis pathway. To investigate the importance of caspases, the pancaspase inhibitor zVAD was used. Hoechst and propidium iodide (PI) staining showed that DHA was able to induce apoptosis independent of caspase 8 and FADD. At 12 h time point DHA (10  $\mu$ M) induced apoptosis in Jurkat A3 as well as in Jurkat caspase 8 and FADD negative cells. Interestingly, zVAD in combination with DHA completely abrogated apoptosis induction and necrosis could be observed (Fig. 22 A). FACS results with annexin/PI staining confirmed the Hoechst and PI data, showing high apoptosis and necrosis induction in Jurkat A3 cells in dose dependent manner in comparison to the ethanol control, reaching significant difference ( $P < 0.001$ ) in response to 40  $\mu$ M DHA. In case of caspase 8 negative cells apoptosis and necrosis were also highly upregulated. Jurkat FADD negative cells were the most resistant in apoptosis and necrosis induction in response to DHA treatment and did not reach significance but in case of the lower concentration of 10  $\mu$ M DHA corresponded to 18 % of cells positive for annexin and PI, reaching significance in comparison to ethanol control ( $P < 0.05$ ) (Fig. 22 B). This result suggested that DHA – induced apoptosis takes place even if the extrinsic apoptotic pathway is blocked. In addition, it also demonstrated that the caspases play a pivotal role in DHA-induced apoptosis as zVAD blocked apoptosis in DHA treated cells.

#### **3.1.5 DHA induces apoptosis in normoxia and hypoxia**

To evaluate the importance of apoptosis for DHA action under normoxic and hypoxic conditions, FACS analyses was performed employing HCT116 colon cancer cells. Apoptosis was quantified by analyzing the amount of cells with low mitochondrial membrane potential (TMRE staining) and the amount of cells with DNA fragmentation (Nicoletti staining).



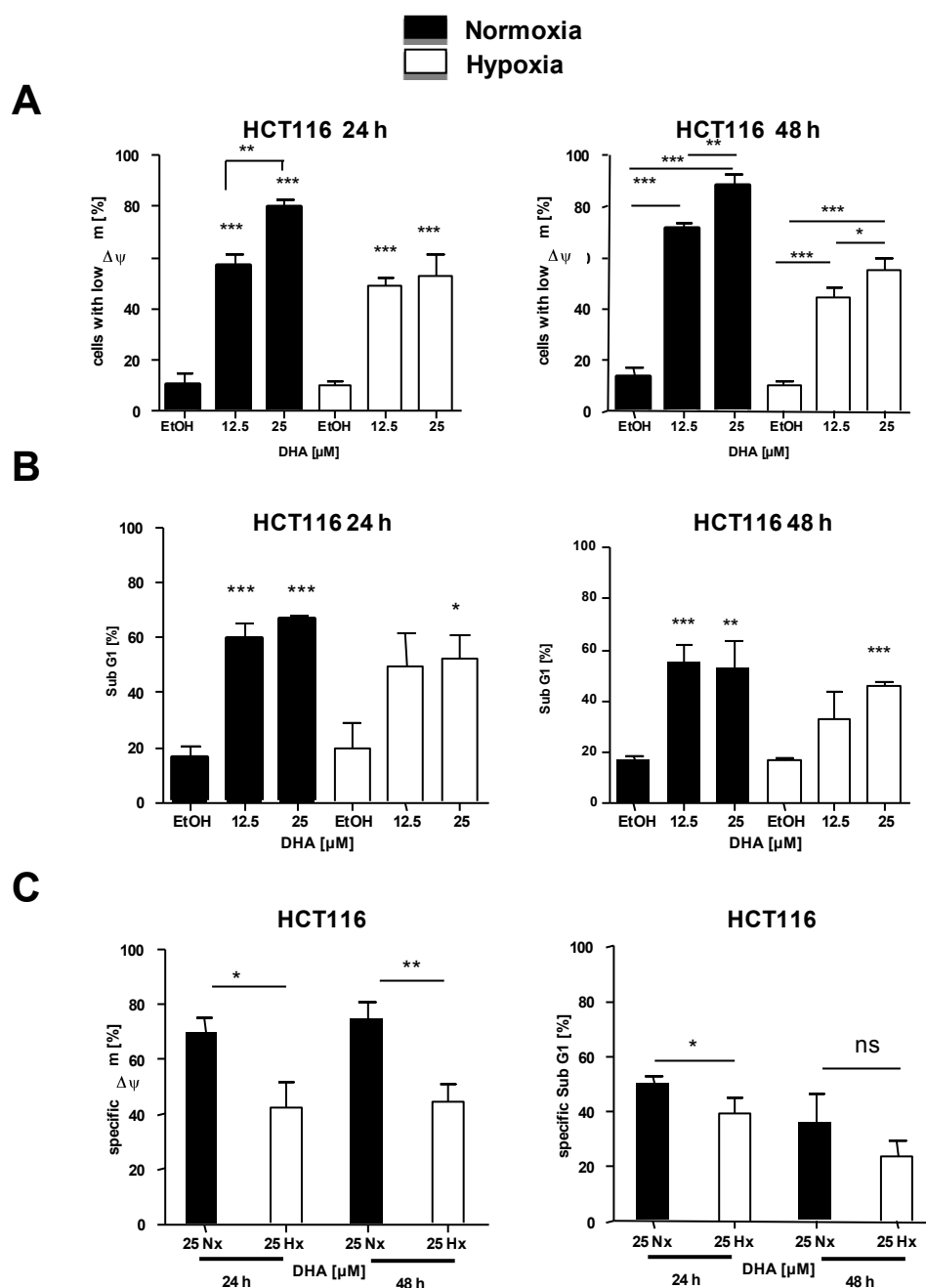


**Fig. 22 DHA induces apoptosis via intrinsic apoptotic pathway.** (A) Jurkat A3, Jurkat FADD<sup>-/-</sup> and Jurkat caspase 8<sup>-/-</sup> T lymphoma cell lines were plated and treated with DHA alone or in combination with 20  $\mu$ M zVAD. 12 h later cells were stained with Hoechst and propidium iodide and were analyzed by fluorescence microscopy. Scale bar corresponds to 100  $\mu$ m (B) Apoptosis was studied by FACS analyses in Jurkat A3, FADD<sup>-/-</sup> and caspase 8<sup>-/-</sup> cells 24 h after DHA treatment (0–40  $\mu$ M). Annexin V and propidium iodide positive stained cells were considered as apoptotic or late apoptotic and necrotic cells. Graph represents means from three independent experiments  $\pm$ SD. Statistical analysis was performed according to unpaired two tailed *t*-test in comparison to untreated controls. (\*) *P* < 0.05; (\*\*) *P* < 0.01; (\*\*\*) *P* < 0.001 and ns=not significant. (Handrick, Ontikatz et al. 2010)

As shown in fig. 23 A, DHA induced significant depolarization of the mitochondrial membrane potential in both normoxia and hypoxia in dose and time dependent manner. However, the level of DNA fragmentation was induced in dose dependent manner and was not further increased at 48 h time point under both conditions (Fig. 23 B).

Cells with low  $\Delta\psi_m$  were significantly increased in normoxia after treatment with 12.5 and 25  $\mu$ M DHA at both 24 and 48 h time points, reaching 80 % (24 h) and 90 % (48 h) of the total cell number at 25  $\mu$ M DHA (*P* < 0.001) in comparison to the ethanol control. In addition, 25  $\mu$ M DHA induced significantly higher amount of

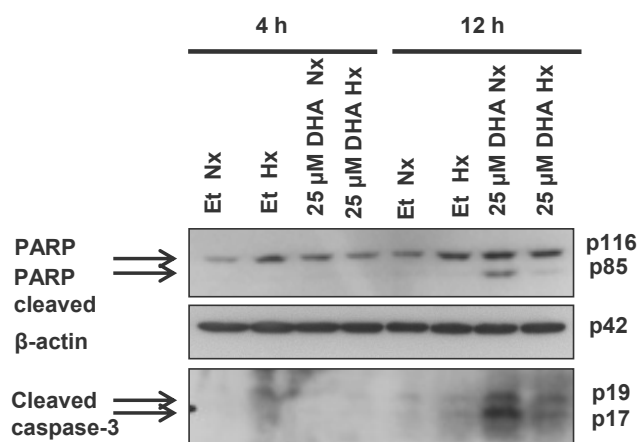
cells with low  $\Delta\psi_m$  in comparison to 12.5  $\mu\text{M}$  DHA ( $P < 0.01$ ) at 24 h (from 60 % to 80 %) and 48 h (from 70 % to 90 %) time points (Fig. 22 A). Cells with low  $\Delta\psi_m$  were also significantly increased under hypoxic conditions in response to DHA treatment at both time points however, were less than under normoxic conditions. DNA fragmentation was significantly increased at 24 and 48 h time points in treated cells with DHA (12.5 and 25  $\mu\text{M}$ ) under both normoxic and hypoxic conditions. DNA fragmentation was already highly induced at 12.5  $\mu\text{M}$  DHA concentration (60 %), however it did not further increase at higher DHA concentration. The same was observed under hypoxic conditions at 24 and 48 h time points (Fig. 23 B). As it can be shown in Fig. 23 C, 25  $\mu\text{M}$  DHA significantly increased the number of cells with low  $\Delta\psi_m$  (apoptosis) under normoxic conditions in comparison to hypoxic conditions at both time points. Similarly, DNA fragmentation was significantly lower under hypoxic conditions in contrast to the normoxic conditions in response to DHA but only at 24 h time point. In case of 48 h time point, DNA fragmentation was lower under hypoxic conditions, however the difference did not reach statistical significance.



**Fig. 23 HCT116 cells are sensitive to DHA-induced apoptosis in normoxia and hypoxia.** HCT116 wt cells were plated and treated with DHA under normoxic (white bars) and hypoxic conditions (black bars). 24 and 48 h later the cells were harvested and analyzed by flow cytometry. **(A)** HCT116 wt cells were analyzed for cells with low mitochondrial membrane potential ( $\Delta\psi_m$ ). For this purpose TMRE staining was used. **(B)** PI in hypotonic citrate buffer was used for staining of DNA content. Sub G1 peak which corresponds to fragmented DNA, was quantified and considered as amount of apoptotic cells. **(C)** The graphs represent the absolute values of treated cells with 25  $\mu$ M DHA corrected with their ethanol controls. The quantifications were performed for  $\Delta\psi_m$  and Sub G1 for both normoxic and hypoxic conditions, at 24 and 48 h time points. The experiments were performed in triplicate. A statistical analysis was performed according to unpaired two-tailed *t*-test. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$  and ns=not significant.

Another characteristic of apoptosis execution is Bax translocation to the mitochondria and the release of cytochrome c from mitochondria to the cytoplasm. Cytochrome c release leads to the caspases activation. To analyze at which time

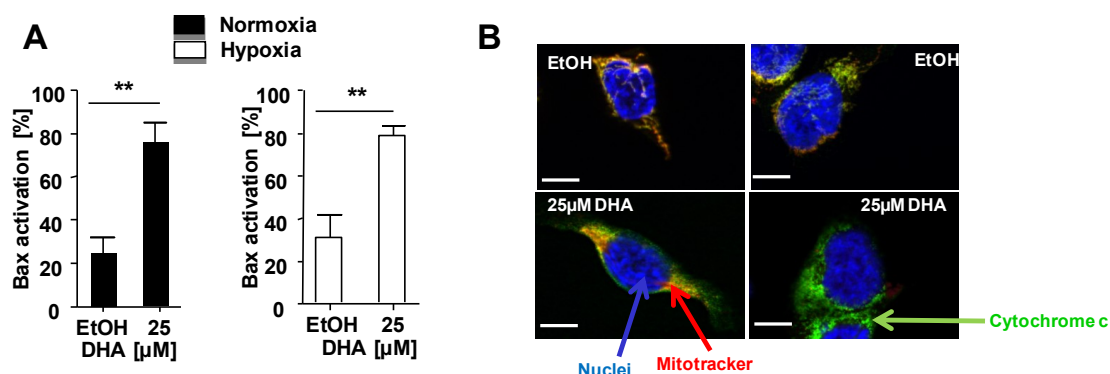
point apoptosis was executed, whole cellular extracts were prepared at different time points (4 h and 12 h) under normoxic and hypoxic conditions (Fig. 24). DHA induced cleavage of caspase-3 and of its substrate, PARP, at 12 h time point in normoxia. In case of hypoxia, DHA induced lower amount of caspase-3 and PARP cleavage at this time point.



**Fig. 24 DHA - induced apoptosis is decreased in hypoxia.** Whole cell lysates of HCT116 treated with DHA in hypoxia and normoxia were analyzed at 4 h and 12 h. For apoptosis detection, specific antibodies which detect cleaved form of caspase 3 and PARP were used in Western Blot. The experiments were done in triplicate and the representative blot is shown.

Due to apoptosis induction at 12 h time point, it was next focused on Bax activation and cytochrome c localization at 12 h under normoxic and hypoxic conditions. Bax activation was monitored using a specific antibody, Bax NT which binds to the activated form of Bax in mitochondria. For the current study FACS analysis was used.

Bax was activated in both normoxic and hypoxic conditions after DHA treatment leading to approximately 90 % of activation (Fig. 25 A). On the other hand, cytochrome c release was monitored by immunofluorescence using a specific antibody. DHA led to cytochrome c release under both normoxic and hypoxic conditions (Fig. 25 B).

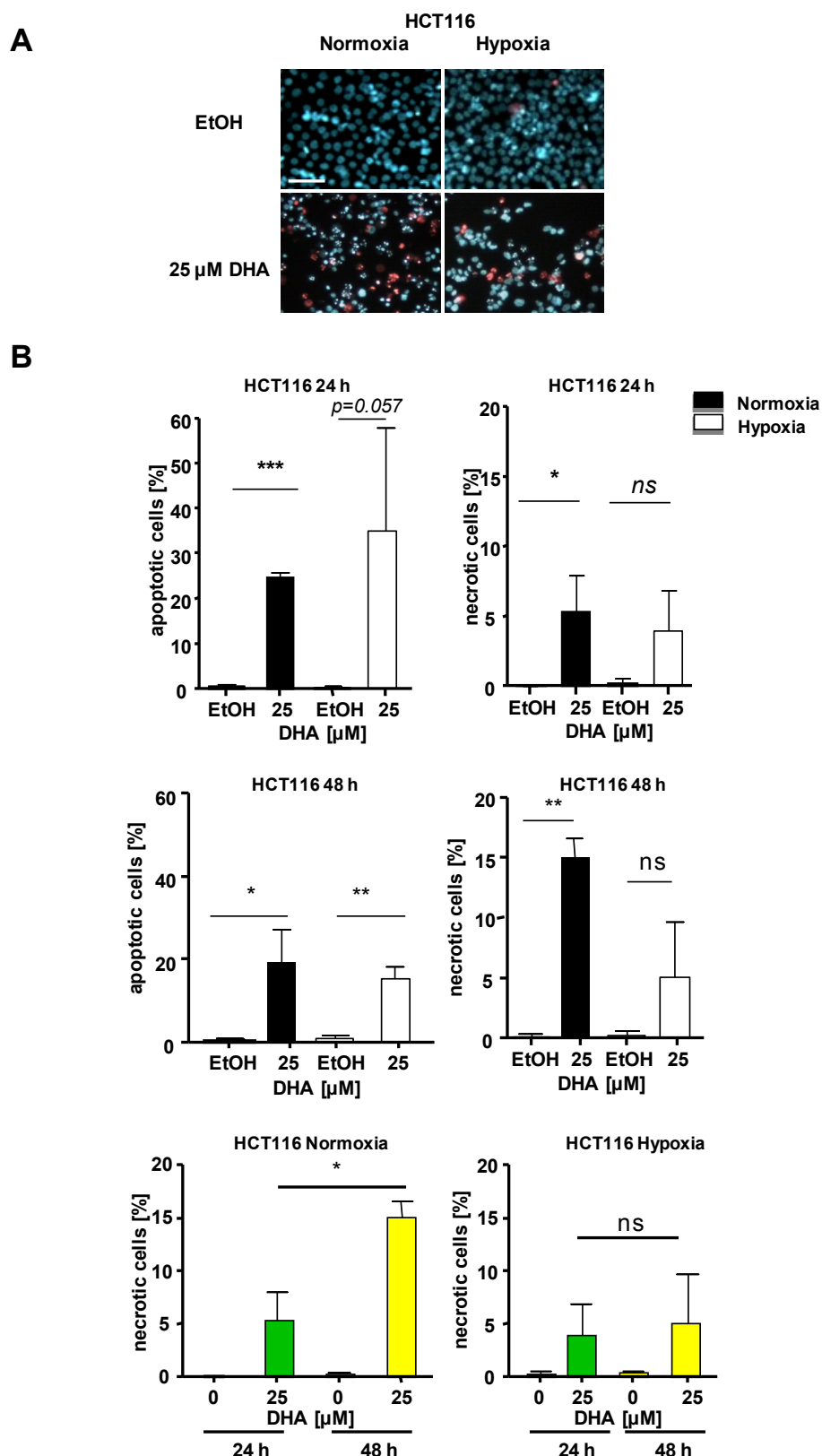


**Fig. 25 DHA induced Bax activation and cytochrome c release in normoxia and hypoxia.** (A) HCT116 cells were treated for 24 h with DHA and analyzed by FACS using an antibody specific for the activated Bax form. The shift of the peak to the right was identified as Bax activation. (B) HCT116 wt cells were treated with 25 μM DHA and 12 h later, cells were fixed and analyzed by immunofluorescence. For cytochrome c detection, primary antibody against cytochrome c was used. Anti rabbit conjugated cy2 antibody was used and subsequently detected by GFP filter. The experiment was performed in triplicate. A statistical analysis was performed according to unpaired two-tailed *t*-test. Results represents means ±SD (\*\*) *P* < 0.01. Scale bar corresponds to 10 μm

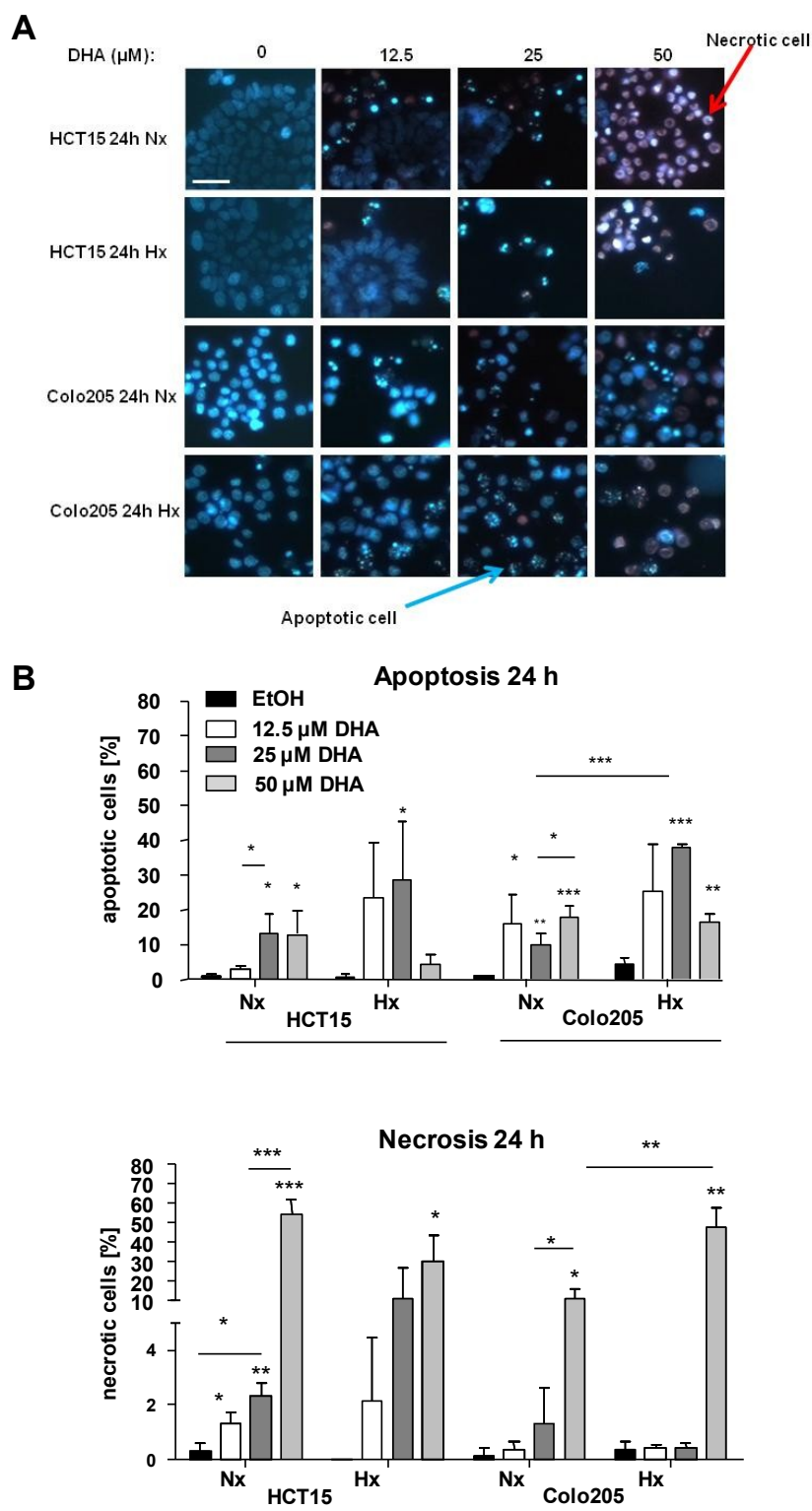
DHA treatment disrupted mitochondrial membrane potential and maybe for this reason, mitotracker was not detectable under hypoxic conditions after DHA treatment, corroborating the results of the TMRE staining (Fig. 23 A). In both ethanol controls in normoxia and hypoxia, cytochrome c was localized into the mitochondria as cytochrome c and mitotracker had same localization.

Further experiments with Hoechst and PI staining were done in order to describe which was the mechanism of death (apoptotic or necrotic).

From evaluations of three independent experiments, it was recognized that DHA induced significant apoptosis and necrosis after 24 h under normoxic conditions. DHA induced further increase in necrosis at 48 h time point reaching significant difference in comparison to the 24 h time point. On the other hand apoptosis and necrosis were not significantly increased after 24h by DHA treatment in hypoxia, but apoptosis was significantly increased after 48 h, thus indicating a delay in DHA action under hypoxic conditions with respect to apoptosis induction. However, DHA did not further increase necrosis after 48 h. This experiment showed that DHA induced apoptosis under both normoxic and hypoxic conditions. However, necrosis was taking place only under normoxic conditions after DHA treatment and was further increased over time. Finally, in hypoxia, DHA induced lower amount of necrotic cells which was not increased over time (Fig. 26).



**Fig. 26 DHA induces apoptotic and necrotic death in HCT116 wt. (A)** HCT116 cells were treated and incubated for 24 h in normoxia or hypoxia. The cells were stained with Hoechst (blue) and PI (red) and visualized by fluorescence microscopy. Scale bar represents 100  $\mu$ m. **(B)** The apoptotic and necrotic cells were quantified at 24 and 48 h time points from three independent experiments. Black bars correspond to normoxia and white bars to hypoxia. Statistical analysis was done by unpaired two-tailed *t*-test. (\*)  $P < 0.05$ ; (\*\*\*)  $P < 0.001$  and *ns*=not significant.



**Fig. 27 DHA induced apoptosis and necrosis in HCT15 and colo205 cells in normoxia and hypoxia.** (A) HCT15 and colo205 cells were plated and treated with DHA (0-50  $\mu\text{M}$ ) under normoxic and hypoxic conditions. 24 h later cells were stained with Hoechst and PI and analyzed by fluorescence microscopy. Representative pictures from one of three independent experiments are shown. Scale bar corresponds to 100  $\mu\text{m}$ . (B) Apoptotic and necrotic cells were quantified from three independent experiments. Graphs represent means  $\pm$  SD. Significance test was performed between the treated and untreated samples as well as between normoxic and hypoxic conditions. Statistical analysis was done by unpaired two-tailed *t*-test. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ . Nx=normoxia and Hx=hypoxia

Apoptosis and necrosis was also studied in HCT15 (p53 mutated cells) and colo205 colon cancer cells. The aim was to test whether DHA acts also in other colon cancer cells in regards to apoptosis and necrosis. To investigate the mechanism of cell death, Hoechst and propidium iodide staining was carried out under normoxic and hypoxic conditions.

As it is shown in Fig. 27, DHA induced significant apoptosis and necrosis in HCT15 cells in both normoxia and hypoxia in a dose-dependent manner. Apoptosis induction was higher in these cells under hypoxic conditions reaching the maximum at 25  $\mu$ M concentration and, at higher dose, necrosis was more prominent. However, at 50  $\mu$ M, DHA led to a higher amount of necrosis in normoxia in comparison to hypoxia.

Apoptosis was induced in colo205 in dose-dependent manner in normoxia and hypoxia but apoptosis was significantly higher in hypoxia (25  $\mu$ M DHA) corresponding to 40 % of counted cells and to 12 % in normoxia. Necrosis was highly increased at 50  $\mu$ M DHA under both conditions, however, necrosis was significantly increased in hypoxia in comparison to the same concentration in normoxia. In detail, necrosis induction under normoxic conditions reached 12 % and in hypoxia 40 % of counted cells.

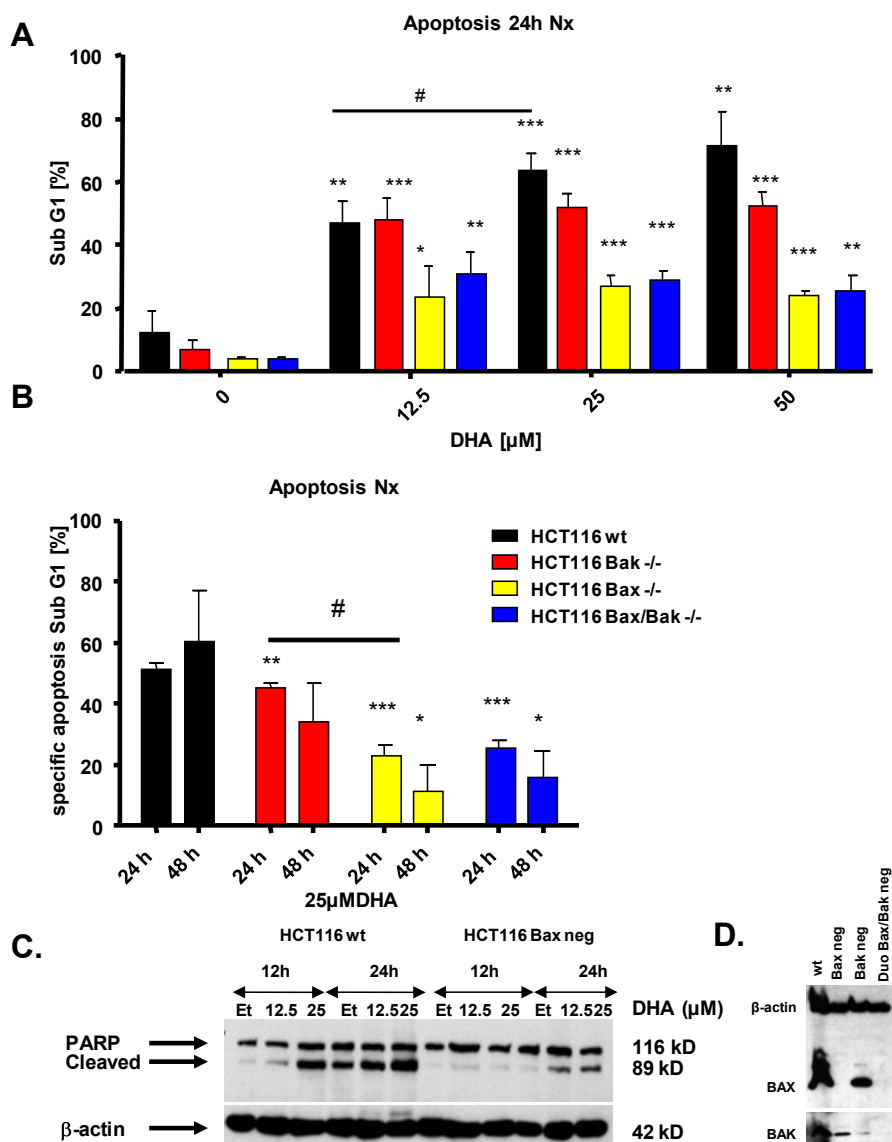
### **3.1.6 DHA – induced apoptosis is Bax/Bak dependent**

To investigate further the intrinsic apoptotic pathway, HCT116 cells with or without expression of Bax or Bak or both were used. Cells were treated with different DHA concentrations for 24 and 48 h under normoxic conditions. At the appropriate time points, cells were harvested and analyzed for apoptosis by flow cytometry using PI staining in low citrate buffer in order to characterize the fragmented DNA (Sub-G1 peak).

DNA fragmentation was increased significantly in cells expressing Bax and Bak in dose-dependent manner and reached 70 % of apoptosis induction in response to 50  $\mu$ M DHA. Especially, Bax was playing important role in apoptosis induction, as the HCT116 Bax negative cells showed decreased apoptosis levels (25 % in response to 50  $\mu$ M DHA) compared to Bax proficient but Bak negative cells (50 % in response to 50  $\mu$ M DHA). Similarly to HCT116 Bax negative cells, the same amount of apoptosis induction was observed in double negative Bax/Bak cells in response to 50  $\mu$ M DHA (Fig. 28 A). DHA did not induce additional apoptosis over the time as it can be seen in Fig. 28 B. Calculating the specific apoptosis in DHA treated cells, it was identified that DHA induced apoptosis in Bax dependent but Bak independent manner (apoptosis induction in response to DHA was increased



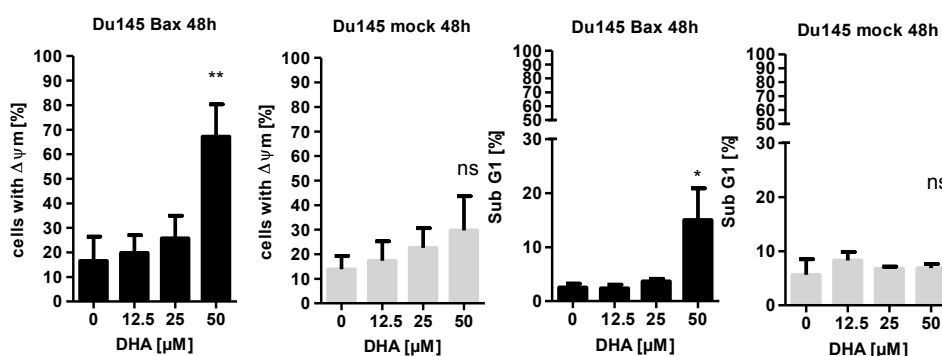
as follows: wt > Bak<sup>-/-</sup> > Bax<sup>-/-</sup> = Bax/Bak<sup>-/-</sup>). HCT116 wt and HCT116 Bax negative cells were further analyzed by Western blotting focusing on apoptotic markers such as PARP cleavage, which is a caspase-3 substrate during the apoptosis. DHA induced PARP activation in dose and time dependent manner and apoptosis was strongly delayed in absence of Bax (Fig. 28 C).



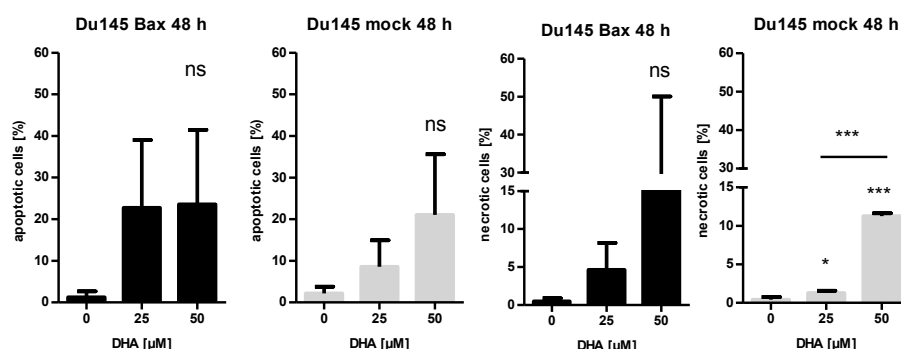
**Fig. 28 Bax and Bak influence apoptosis induction after DHA treatment.** (A) HCT116 cells which were either wt or Bax or/and Bak negative, were treated with different DHA concentrations. 24 h later the cells were analyzed for apoptosis by flow cytometry. (B) Specific apoptosis was calculated by extracting ethanol control. Graph represents the specific apoptosis from FACS analyses for 24 h and 48 h time points. (C) HCT116 wt and HCT116 Bax negative cells were plated and treated with increasing dose of DHA and incubated for 12 and 24 h. Whole cell lysates were performed and analyzed for caspase activation by Western blotting. (D) Expression of pro-apoptotic proteins Bax and Bak were tested by Western blotting analyses. Experiment for FACS analyses and western blots were performed in triplicate. For Western blots representative pictures are shown. A statistical analysis was performed according to unpaired two-tailed *t*-test. (\*) *P* < 0.05; (\*\*) *P* < 0.01; (\*\*\*) *P* < 0.001.

Importance of Bax and Bak in DHA-induced apoptosis was shown also in Du145 prostate cancer cells which do not express p53. Du145 mock cells which are Bak positive but Bax negative showed no significant apoptosis induction (Sub G1 and  $\Delta\psi_m$ ) in response to DHA treatment. In contrast to the Du145 mock cells, the Du145 Bax positive cells showed significant apoptosis in DHA treated cells. Especially, high dose of DHA (50  $\mu\text{M}$ ) led to a significant amount of cells with low mitochondrial membrane potential (70 % of cells with low  $\Delta\psi_m$ ) and to a significant DNA fragmentation at the same DHA concentration (15 % of cells with fragmented DNA) (Fig. 29 A). From these results, it could be concluded that Bax and not Bak are responsible in DHA-induce apoptosis in both HCT116 cells and in Du145 cells. To confirm the result and to discriminate between apoptosis and necrosis induction in response to DHA, DU145 cells were used for apoptosis and necrosis detection via Hoechst and PI staining.

## A



## B

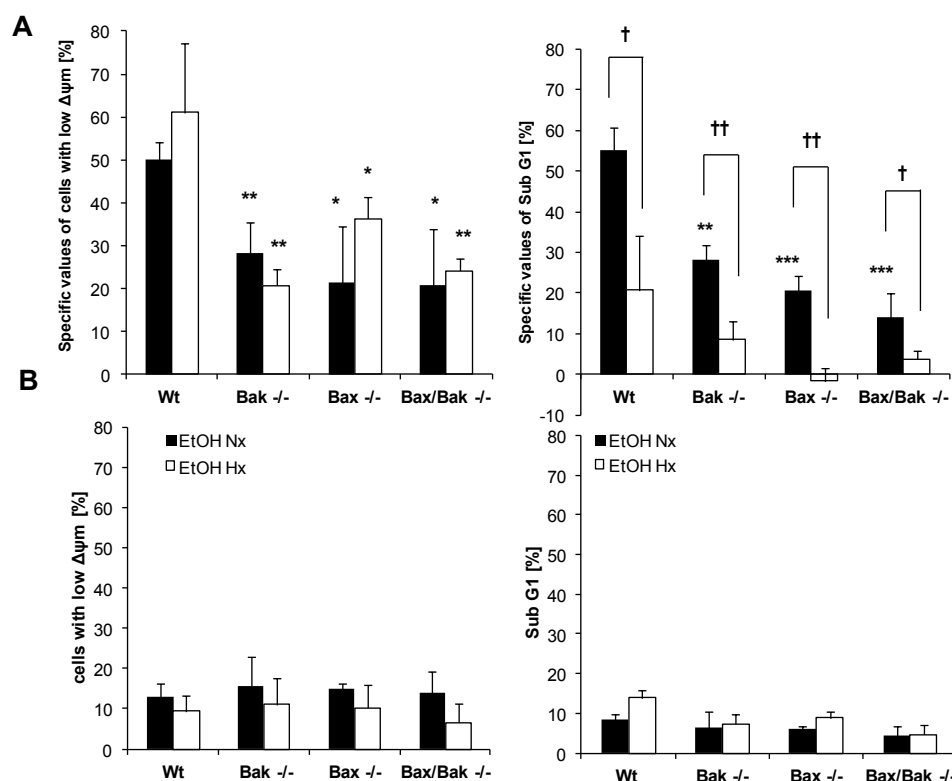


**Fig. 29 DHA-induced apoptosis is Bax dependent.** (A) Du145 mock and Du145 Bax positive cells were treated with DHA and 48 h later cells were harvested and analyzed for apoptosis with FACS analysis and (B) Du145 mock and Du145 Bax positive cells were treated with DHA and 48h later were stained with Hoechst/PI. Graphs represent the quantifications of apoptotic and necrotic cells. The experiments were performed at least three times. A statistical analysis was performed according to unpaired two-tailed *t*-test. Results represents means  $\pm$ SD (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$  (\*\*\*);  $P < 0.001$  and ns. not significant.

In the absence of Bax, DHA induced significant death by necrosis ( $P < 0.001$ ). However, in presence of Bax, DHA was inducing predominantly apoptosis. (Fig. 29 B). To summarize, Bax and not Bak seemed to be essential in apoptosis induction in response to DHA in the colon cancer cells. Loss of Bax led to increased necrotic death in DHA response in Du145 model.

### 3.1.7 DHA-induced apoptosis depends on Bax/Bak also under hypoxic conditions

To further investigate the importance of the proapoptotic Bcl2- proteins Bax and Bak, HCT116 cells which are either lacking Bax or Bak or both were treated with 25  $\mu$ M DHA for 24 h under normoxic and hypoxic conditions. Apoptosis was analyzed using FACS ( $\Delta\psi_m$  and Sub G1) and fluorescence microscopy (Hoechst and propidium iodide staining).



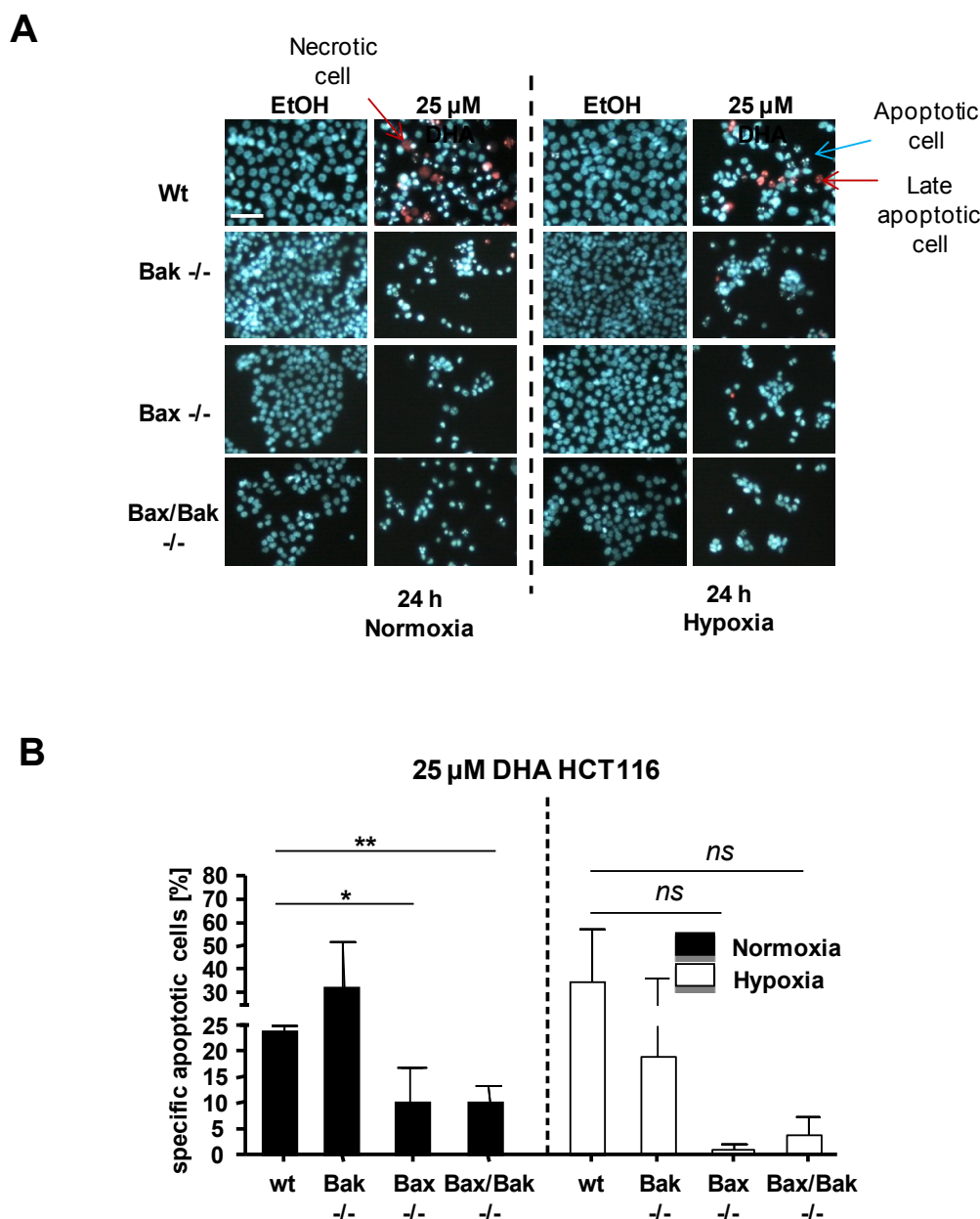
**Fig. 30 Apoptosis is significantly reduced under hypoxic conditions in a Bax/Bak dependent manner.** (A) HCT116 wt, Bax negative, Bak negative, and Bax/Bak negative cells were treated with 25  $\mu$ M DHA in normoxia and hypoxia for 24 h. Apoptosis was monitored with FACS analysis (SubG1 and  $\Delta\psi_m$ ). (B) Graphs represent the controls from the same experiments under normoxic and hypoxic conditions. A statistical analysis was performed according to unpaired two-tailed  $t$ -test. Results represent means of specific values  $\pm$ SD (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ . (\*) was used for comparison between different cells to the HCT116 wt and (†) was used to compare normoxic to the hypoxic conditions.

At the level of the mitochondria, DHA did not show significant differences between normoxic and hypoxic conditions. However, significant differences were observed between the HCT116 wt and the other cell clones (HCT116 Bak <sup>-/-</sup>, HCT116 Bax <sup>-/-</sup>, and HCT116 Bax/Bak <sup>-/-</sup>) under both normoxic and hypoxic conditions. DHA-induced depolarization of the mitochondrial membrane potential required Bax/Bak presence. In case of loss of Bax or/and Bak, cell with low  $\Delta\psi_m$  were significantly reduced in response to the same concentration of DHA (25  $\mu$ M). Interestingly, under hypoxic conditions Bak but not Bax showed to play an important role in induction of mitochondrial depolarization. HCT116 Bak <sup>-/-</sup> and HCT116 Bax/Bak <sup>-/-</sup> cells showed to induce similar result in depolarization of the mitochondria. However, HCT116 Bak <sup>-/-</sup> cells did not reach significant difference in comparison to the HCT116 wt. At the level of DNA fragmentation, in contrast to the normoxic conditions, DHA showed significant reduction in Sub G1 under hypoxic conditions for all HCT116 clones. In addition Bax and Bak played a pivotal role in DHA-induced DNA fragmentation as shown in Fig. 30 A right panel, where the Sub G1 is significantly lower in Bak or/and Bax negative cells in comparison to the HCT116 wt. In hypoxia Bax and Bak showed also an importance in DHA-induced apoptosis, however the difference between the wt and the HCT116 clones did not reach statistical significance. Finally, in case of DNA fragmentation Bax presence showed to be responsible for apoptosis induction in response to DHA. Loss of Bax resulted to show similar amount of cells in Sub G1 compared to the HCT116 Bax/Bak negative cells (20 and 15 % respectively). The ethanol treated cells were not showing changes in the amount of apoptosis under both normoxic and hypoxic conditions (Fig. 30 B).

Hoechst and PI staining was performed in order to examine the mechanism of death induced by DHA in HCT116 wt, HCT116 Bak or and Bax negative. Cells were plated in 12-well plates and treated in normoxia or hypoxia with 25  $\mu$ M DHA. 24 h later the cells were stained and analyzed by fluorescence microscopy.

DHA induced apoptosis in all cell lines, however apoptosis induction was significantly reduced when Bax or Bak were missing. Necrosis was observed only in HCT116 wt and it was not detectable in other cell lines (Fig. 31 A).

From the Hoechst and PI staining results it was possible to confirm that DHA induced apoptosis in a Bax dependent manner. In addition, apoptosis was reduced under hypoxic conditions in all the cell lines however it was still Bax dependent (Fig 31 B) but it did not reach significant difference in normoxia. Finally, necrosis was highly induced in HCT116 wt as shown in Fig. 26 B, and not observed in cell lines lacking Bax/Bak.

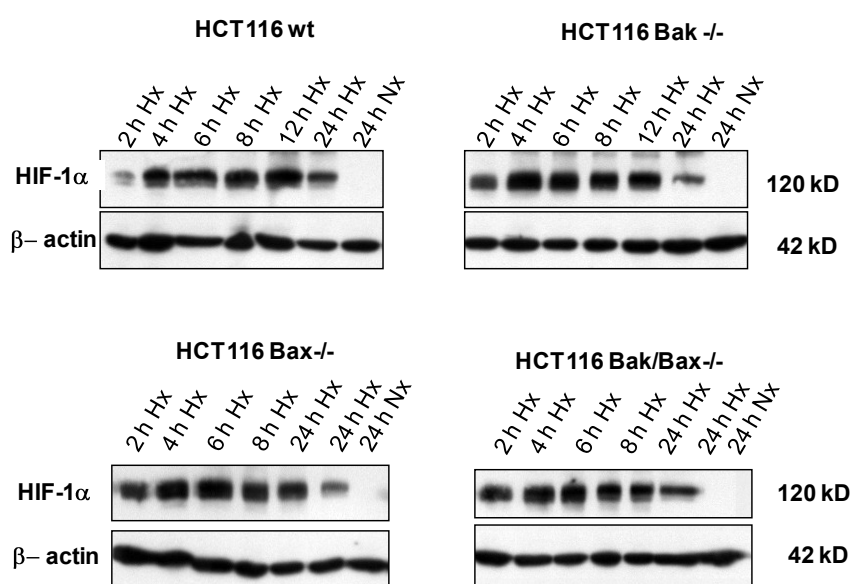


**Fig. 31 DHA induces cell proliferation down-regulation and apoptosis induction in normoxia and hypoxia in Bax/Bak dependent manner. (A)** HCT116 wt, Bax or/and Bak negative. cells were treated with 0-25  $\mu$ M DHA in normoxia or hypoxia. 24 h later cells were stained with Hoechst and PI. Apoptosis and necrosis were identified by fluorescence microscopy. Representative pictures are shown. Scale bar corresponds to 100  $\mu$ m **(B)** Apoptotic cells were quantified. Graph represents means from absolute values for apoptotic cells from three independent experiments  $\pm$  SD. The experiment was done in triplicate Statistical analyses was performed according to unpaired two-tailed *t*-test. (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ , *ns*= not significant.

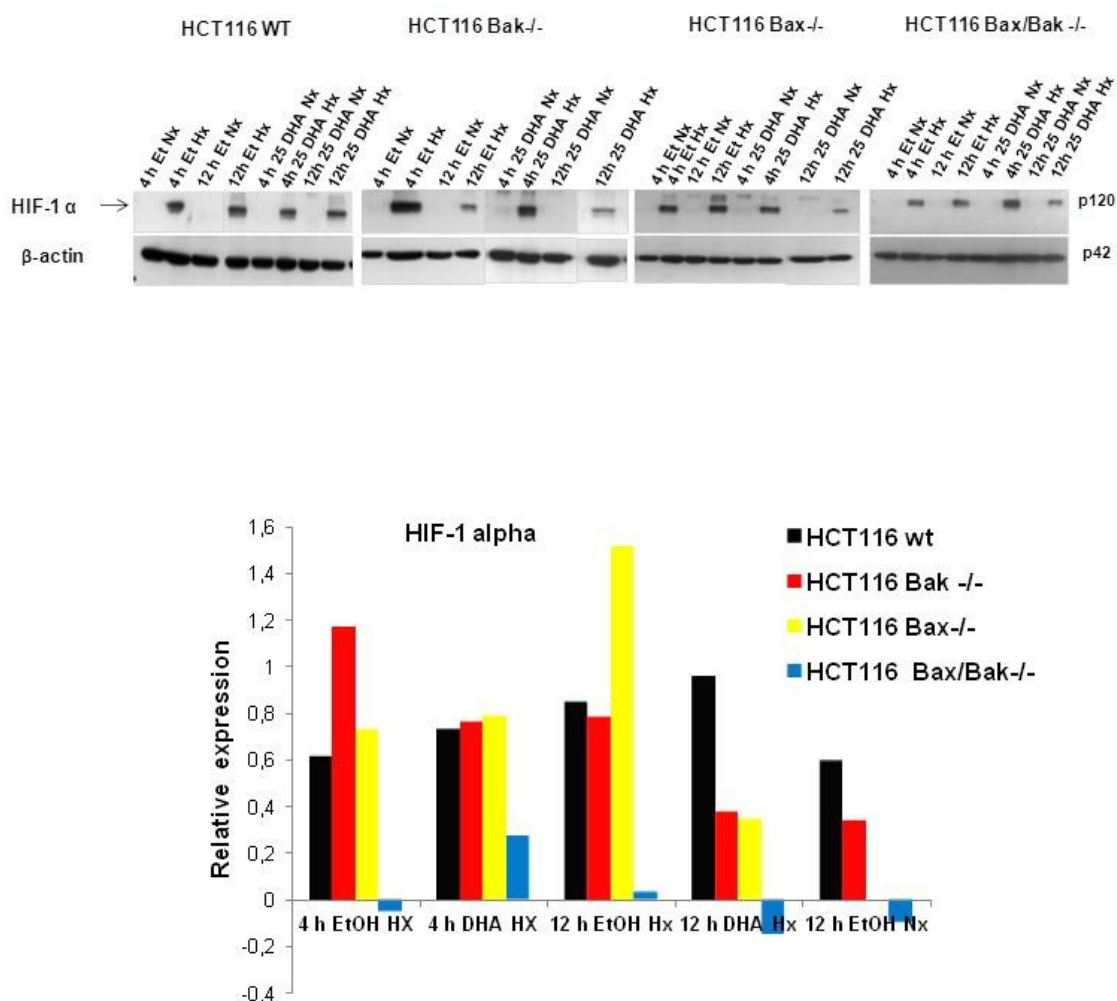
### 3.1.8 DHA induces reduction of HIF-1 alpha expression in the absence of Bax

In cancer, both hypoxia and mutations in oncogenes as well as in tumor suppressor genes increase HIF-1 activity. HIF-1 target genes have been identified, and many of these are associated with angiogenesis, invasion, and metastasis (Semenza 2003). Considering this, it was investigated whether DHA could induce downregulation of HIF-1 alpha expression at the protein level. For this purpose the HCT116 cell clones were used. First, HCT116 wt, HCT116 negative for Bax or Bak and HT116 Bax/Bak negative cells were analyzed for HIF-1 $\alpha$  relative expression over the time in an environment containing 0.2% of O<sub>2</sub> (Fig. 32). The Western-blot analysis showed that all the HCT116 cell clones do not express HIF-1 $\alpha$  under normoxia but only under hypoxia. Under hypoxia, HIF1 $\alpha$  protein level increased in a time dependent manner in all the cell lines. For all the cell lines HIF-1 $\alpha$  protein levels were decreased after 24 h in hypoxia (Fig. 32)

The same experiment was performed with DHA treated cells compared to the ethanol controls. At early time points (4 h), DHA induced no changes in HIF-1 $\alpha$  expression in comparison to the ethanol control. In contrast to the 4 h time point, at 12 h time point, DHA down-regulated HIF-1 $\alpha$  expression in Bax or Bak negative cells. The drug did not influence the HIF-1 $\alpha$  expression in HCT116 wt cells (Fig. 33).



**Fig. 32 HIF-1 $\alpha$  expression over the time in HCT116 cells.** HCT116 wt, Bak<sup>-/-</sup>, Bax<sup>-/-</sup> and Bak/Bax<sup>-/-</sup> cells were treated with hypoxia for different time points (0-24h) and whole cell extracts were performed. HIF-1 $\alpha$  expression was analyzed by Western blots. Experiment was performed two times and representative blot is listed.



**Fig. 33 DHA downregulates HIF-1α protein expression, in response to loss of Bax/Bak.** HCT116 wt, Bak neg, Bax neg. and Duo Bax/Bak neg. cells were treated with DHA or ethanol for 4 and 12 h under hypoxic conditions. 12 h ethanol control was used as a negative control for HIF-1α expression under normoxic conditions. Densitometric analysis of the current blot and the ratio of HIF-1α/β-actin were calculated and used for the graph. The experiment was performed two times and the representative blots were used.

### 3.1.9 DHA is cytostatic and cytotoxic for HCT116 cells

From previous results in HCT15, colo205 and HCT116 wt cell lines, it was shown that DHA induces significant cell proliferation inhibition and reduces the viability of the treated cells under normoxic and hypoxic conditions (Fig. 17-Fig. 20). Therefore it was important to analyze whether DHA treatment might induce reduction in cell viability and inhibition of cell proliferation in Bax/Bak dependent manner. To this aim, the viability and proliferation capacity of HCT116 cells, which were either wild type or Bax or/and Bak negative, were analyzed by WST-1 assay (Fig 34) and by trypan blue staining (Fig. 35).

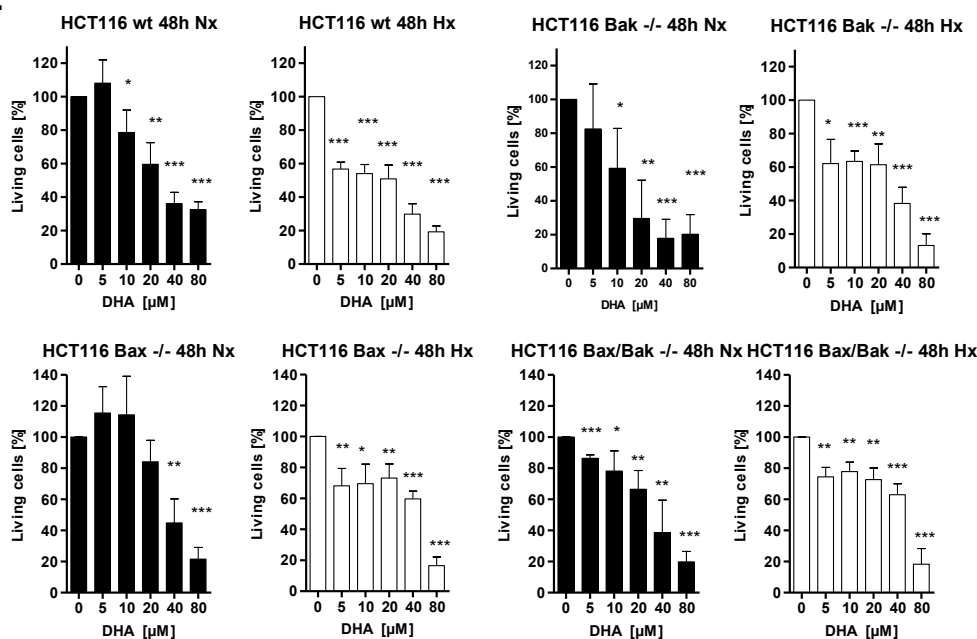
WST-1 assay using HCT116 wt, Bax negative, Bak negative or Bax/Bak double negative cells, was performed in normoxia and hypoxia and the

experiment was stopped after 24 h or 48 h. Fig. 34 A represents graphs with the percentage of values normalized by the ethanol controls at 48 h time point.

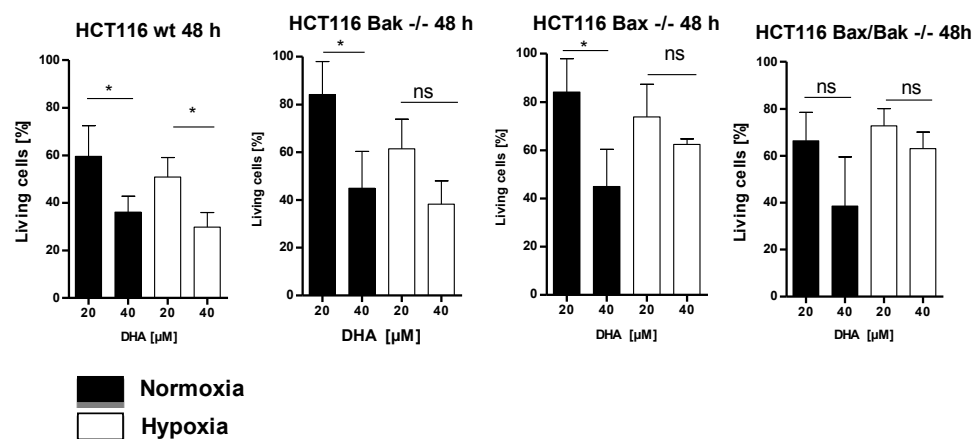
The results showed that, in normoxia, DHA induces reduction of living cells in dose dependent manner and it is influenced by the loss of Bak or Bax. Indeed, HCT116 wt showed strong reduction of viable cells in dose dependent manner and the maximum effect was achieved at 40  $\mu$ M DHA which corresponded to 30 % of living cells in normoxia. Instead, under hypoxic conditions, DHA induced strong proliferation inhibition and only at higher concentrations of 40 and 80  $\mu$ M, DHA induced further reduction of cell viability (20 % of viable cells at 80  $\mu$ M DHA). Compared to the wt cells, the Bak negative cells were less sensitive under both normoxic and hypoxic conditions. In normoxia cell viability showed similar pattern of concentration dependency as for the HCT116 wt. At the higher concentrations (40 and 80  $\mu$ M DHA), DHA treatment achieved the same OD values (viability) which corresponded to 20 % of viable cells. In hypoxia, DHA induced mostly cell proliferations inhibition at lower DHA concentration until 20  $\mu$ M and at higher DHA concentrations, such as 40 and 80  $\mu$ M, cell viability was reduced to 15 %. HCT116 Bax negative or HCT116 Bax/Bak negative cells showed to be more resistant to DHA under both normoxic and hypoxic conditions compared to the HCT116 wt and the Bak negative cells. As it is demonstrated in Fig 34 B, there were no significant differences observed between the normoxic and hypoxic conditions in cell viability in response to 20 and 40  $\mu$ M DHA in all the tested cell lines. However, cell viability was significantly reduced in dose dependent manner under both normoxic and hypoxic conditions in HCT116 wt. In case of Bax negative or Bak negative cells, the significant difference was only observed in normoxia but no longer in hypoxia. In addition the HCT116 duo Bax/Bak negative cells, showed to be the most resistant and did not reach significant difference in viable cells between the 20 and 40  $\mu$ M DHA under both conditions.



A.



B.



■ Normoxia  
□ Hypoxia

C.

DHA [μM]	HCT116 wt	HCT116 Bak -/-	HCT116 Bax -/-	HCT116 Bax/Bak -/-
LC50 Nx	21,5	24	35,3	38
LC50 Hx	8	64,8	71	62
STDV Nx	4,7	10,6	5,5	16,3
STDV Hx	6	11,2	22	6,5

**Fig. 34 DHA induces proliferation inhibition to HCT116 model in normoxia and hypoxia. (A)** HCT116 cells were plated in 96 well plates and treated in normoxia or hypoxia. 48 h later the absorbance of the supernatant was measured by ELISA reader after 1 h with WST-1 compound. Graphs show the percent of absorbance compared to untreated cells. The experiments were made in triplicate. **(B)** The percent of the OD values (living cells) were compared to the percent of the hypoxic OD values for 20 and 40 μM DHA **(C)** The LC50 of DHA in HCT116 were calculated from the formula with four parameters:  $LC_{50}: y = (a-d)/(1+(x/c)^b) + d$  given from the fitting of the curve by KC4 software. All the experiments were performed in triplicate  $\pm$  SD. Statistical analysis was performed according to unpaired two-tailed *t*-test (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ ; ns=not significant.

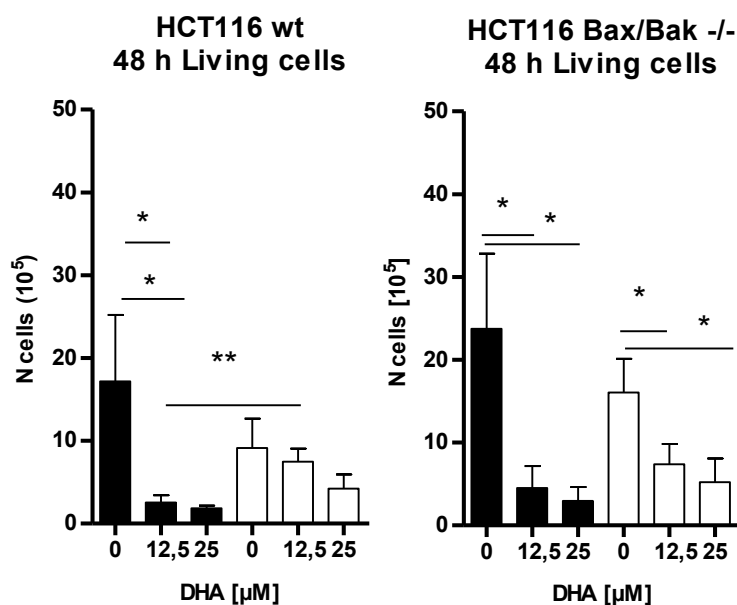
Finally, the LC50 values were calculated and it was observed that HCT116 wt was the most sensitive cell line to DHA-induced cell death (reduction of viable cells) under both normoxic and hypoxic conditions. Interestingly, under hypoxic conditions HCT116 wt were even more sensitive to DHA treatment (8  $\mu$ M DHA). Loss of Bak or/and Bax, showed significant resistance to the DHA-induced death and it was even higher under hypoxic conditions (Fig. 34 C). In hypoxia, loss of Bak and Bax causes higher LC50 values of DHA concentration (ca. 60  $\mu$ M).

These results suggest that Bax is essential to induce reduction of cell viability in HCT116 cells under normoxic conditions; however, Bax and Bak are needed under hypoxic conditions to induce cell death. DHA is cytotoxic in normoxia and cytostatic in hypoxia in HCT116 cells.

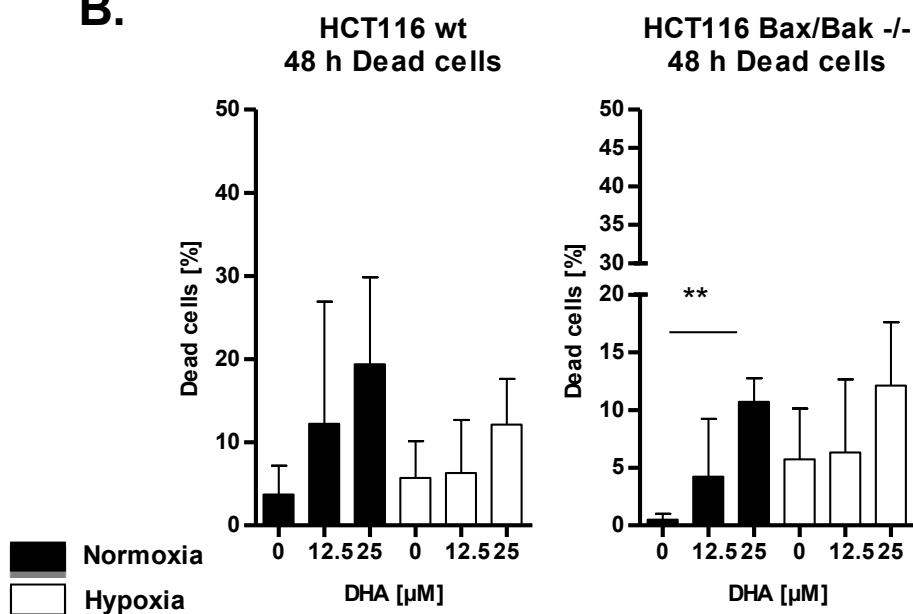
To corroborate our findings and to exclude potential artifacts of the WST-1 assay under hypoxic conditions, a trypan blue test was performed using HCT116 wt and HCT116 Bax/Bak negative cells (Fig. 35). The cells were stained with trypan blue and at the 48 h time point; cells were counted under the microscope.

Treatment with DHA inhibited cell proliferation in HCT116 wt and Duo Bax/Bak negative clones under normoxic conditions. Hypoxia treatment alone showed also an impact in cell proliferation in both cell lines (cells in hypoxia were proliferating slowly). However, the effect was not significant (Fig. 33 A). DHA did not further diminish cell proliferation in the HCT116 wt, and was not significantly reduced in comparison to its control under hypoxic conditions. Moreover, treatment of HCT116 Bax/Bak negative cells induced significant down-regulation in cell proliferation in comparison to the control in hypoxia. Moreover, DHA did not induce significant increase of the dead cells in HCT116 wt cells under both normoxic and hypoxic conditions. In contrast, Bax/Bak negative cells showed a significant increase in dead cells in response to DHA in normoxia, but not in hypoxia (Fig. 35 B).

A.



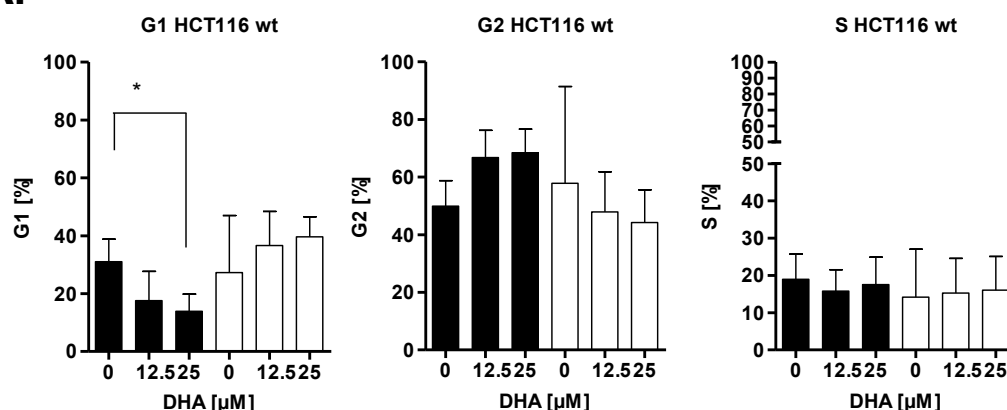
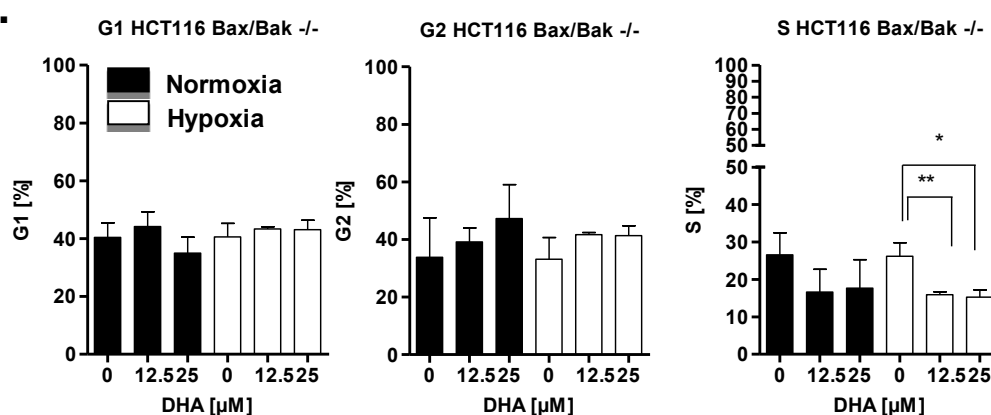
B.



**Fig. 35 DHA was cytotoxic for HCT116 wt and HCT116 Bax/Bak -/- cells in normoxia and hypoxia. (A)** Cells were plated ( $2 \times 10^4$ ) and treated with DHA (0-25  $\mu$ M) in normoxia (black bars) and hypoxia (white bars) and after 48 h cells were harvested and stained with trypan blue in order to discriminate between the living and the dead cells. Cells were counted under the microscope. The intact cells which were not positive for trypan blue staining were characterized as living cells. The graphs contain the absolute numbers of cells counted from three independent experiments. **(B)** Trypan blue stained cells were characterized as dead cells. The graphs represent the percentage of dead cells. The graphs represent means  $\pm$ SD from triplicate experiments. Statistical analyses was performed according to unpaired two-tailed *t*-test (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ .

In order to investigate whether DHA-induced cell proliferation inhibition was due to cell cycle arrest, cell cycle regulation was further investigated. For these experiments, the cell cycle distribution of HCT116 wt and HCT116 Bax/Bak negative cells was analyzed by flow cytometry. The cells were treated in normoxia and hypoxia and 24 h later they were analyzed for the cell cycle distribution. A FACS analysis was performed with propidium iodide staining in hypotonic citrate buffer. The results from three independent experiments are represented in Fig. 36.

Results from the FACS analyses showed a significant decrease in G1 phase in HCT116 wt in response to DHA under normoxic conditions in comparison to the ethanol control. The effect was altered by an increase in the number of cells in G2/M phase however, didn't reach a significant difference. Hypoxia alone did not influence the cell cycle distribution in comparison to normoxia in HCT116 wt cells. Under hypoxic conditions HCT116 wt cells were increased in G1 phase in response to DHA, however not significantly. The G1 increase was leading to a small decrease in G2 phase, and S phase was not influenced in response to DHA treatment. In case of HCT116 Bax/Bak negative cells, no significant change in G1 was observed and only a small increase in G2 phase was detected in normoxia. In addition, G1 and G2 phase were also not changed in normoxia. DHA significantly dropped the S phase under hypoxic conditions. Hypoxia alone did not influence the cell cycle distribution in comparison to the normoxic conditions.

**A.****B.**

**Fig. 36 DHA cause cell cycle arrest in HCT116 clones. HCT116 wt and HCT116 Bax/Bak negative cells were plated and treated with DHA.** At 24h time point, cells were harvested and analyzed for the cell cycle distribution by FACS. The quantifications from the different cellular phases from FACS analyses under normoxic and hypoxic conditions were taken according to histogram plots from three independent experiments. Graphs represent the percentage from different phases of cell cycle in HCT116 wt (**A**) and HCT116 Bax/Bak negative cells (**B**). The apoptotic cells were excluded from the measurements (G1+S+G2 phases were considered to 100 %). The graphs represent means  $\pm$ SD from triplicate experiments. Statistical analysis according to unpaired two-tailed *t*-test (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ .

### 3.1.10 Bax and Bak are critical for DHA – induced death of clonogenic tumor cells in normoxia but not in hypoxia

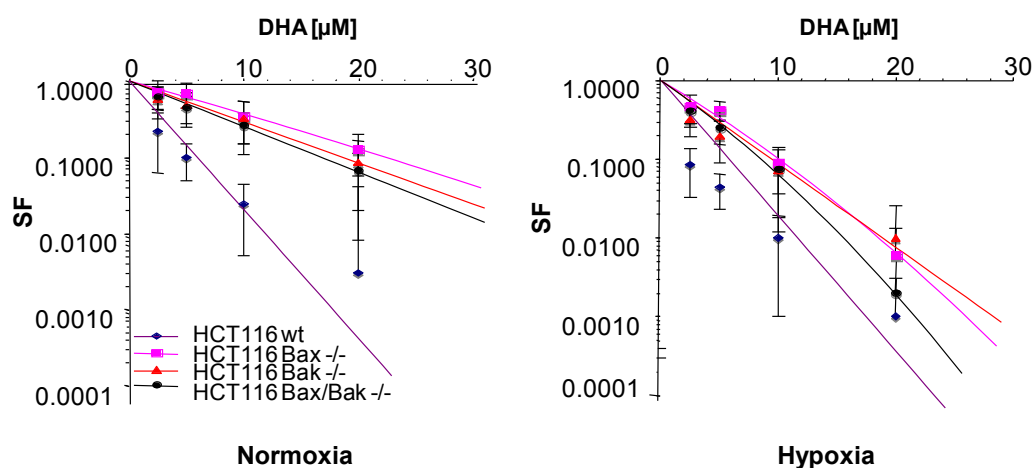
All the experiments which were described until now were short term assays which, differently from long term assays such as the colony formation assay, have limited clinical relevance. A clonogenic assay is a technique to study the effectiveness of specific agents on the survival and proliferation of cells. It is especially important in cancer research laboratories to determine the effect of drugs or radiation on proliferating tumor cells (Hoffman 1991).

In a first part, the addressed question was whether DHA could induce clonogenic death under normoxic and hypoxic conditions to be able to

subsequently identify the importance of the pro-apoptotic proteins, Bax and Bak in clonogenic death induction.

Thus, in the current study, the antineoplastic efficacy of DHA in HCT116 colon cancer cells under normoxic and hypoxic conditions was examined. HCT116 wt, HCT116 Bak negative, HCT116 Bax negative and HCT116 Bax/Bak negative cells were plated for colony formation assay. In case of hypoxia, the cells were preincubated in a hypoxia chamber for 2 h treated with DHA and after 24 h the treated and control cells were further incubated under normoxic condition for other 10 days, until the colonies were formed.

The results from three independent experiments were analyzed and the survival fractions were calculated (Fig. 37). As shown in the graph, DHA induced clonogenic death in dose dependent manner in all the cell clones under both normoxic and hypoxic conditions. The results demonstrate that Bax and Bak are required to signal the clonogenic death in response to DHA treatment. The cells which were lacking either Bax or Bak or both were more resistant to DHA-induced death of clonogenic tumor. Interestingly, under hypoxic conditions the cells were sensitive to DHA-induced death and this result was Bax/Bak independent. That suggested that DHA might act using alternative mechanisms in order to induce clonogenic death.



**Fig. 37 DHA induces clonogenic death in normoxia and hypoxia.** HCT116 cells were plated for colony formation assay and treated with 0-20  $\mu\text{M}$  DHA under normoxic or hypoxic conditions. After 24 h in hypoxia, corresponding plates were further incubated in normoxia. The experiment was stopped after 10 days of incubation. The surviving fraction (SF) was calculated from counted colonies (>50 cells) divided with plated number of cells. Values were corrected by plating efficiency of the cells treated with ethanol.

### 3.1.11 DHA induces autophagy in HCT116 cells

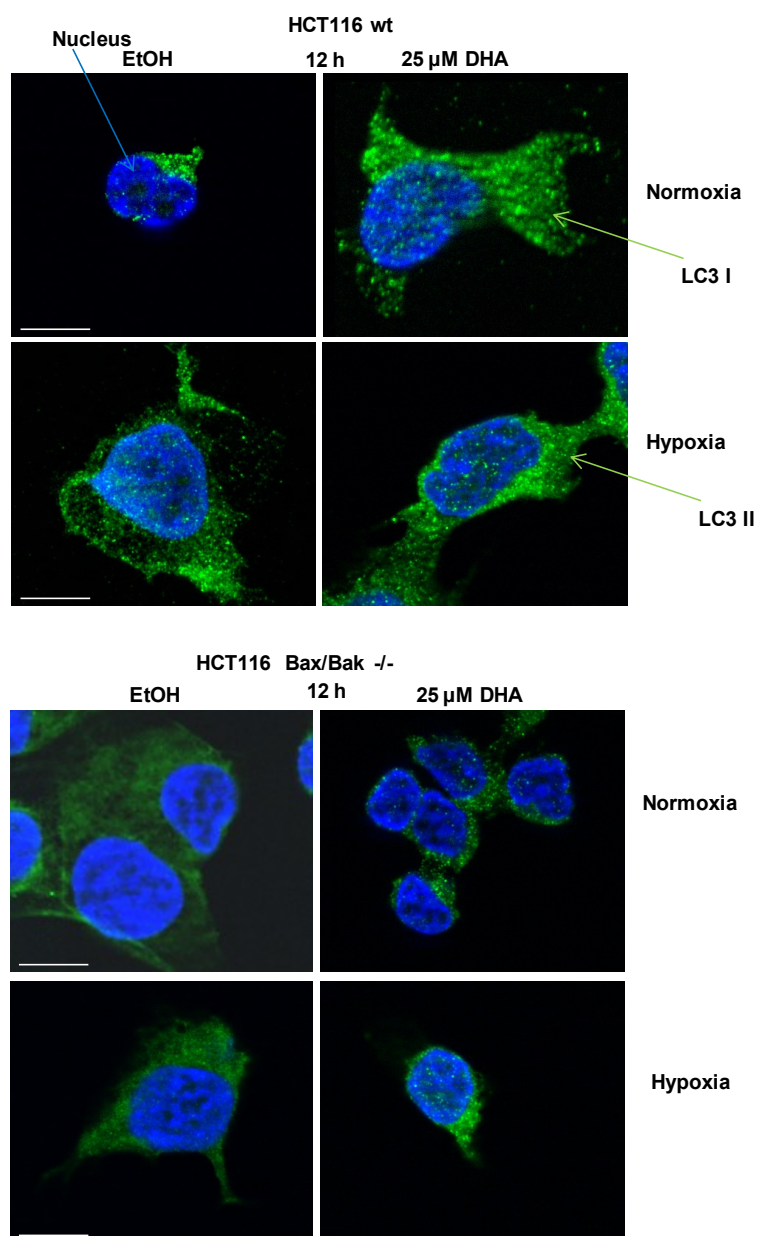
The results obtained from colony formation assay led to the hypothesize that DHA may induce Bax/Bak independent clonogenic death by using an alternative death mechanism in hypoxia. Autophagy was then examined as an alternative death mode in response to DHA treatment. To investigate whether DHA induced autophagy, HCT116 wt and HCT116 double Bax/Bak negative cells were used in the following experiments in normoxia and hypoxia.

To study the autophagy, microtubule-associated protein1 light chain 3 (MAP-LC3 or LC3) specific antibody was used as a marker for autophagy induction. The localization of LC3 in the specific organelles autophagosomes is an indicator for cells undergoing autophagy. During the process LC3 is cleaved and LC3-II translocates into the autophagosomes. Therefore, immunofluorescence staining was used to identify the localization of LC3 in the cells after treatment with DHA.

The result from the immunofluorescence experiments showed that HCT116 wt cells had similar autophagic behavior in normoxic and hypoxic conditions. The untreated cells (ethanol controls) showed basic levels of autophagy and after DHA treatment, autophagy was slightly increased. However, it was not significantly increased in comparison to the ethanol solvent control from counting the punctuated structures which correspond to the autophagic vacuoles.

In contrast to the wild type, the Bax/Bak negative cells did not show any autophagy in ethanol control in normoxia and hypoxia, however autophagy was observed in response to 25  $\mu$ M DHA treatment in both conditions (Fig. 38).

LC3 localization in autophagosomes increased in HCT116 wt cells under both conditions. In contrast, a cytoplasmic LC3 localization was found in HCT116 Bax/Bak negative cells in normoxia and hypoxia. DHA induced significant increase in LC3 levels in autophagosomes in both HCT116 wt and Bax/Bak negative cells under normoxic and hypoxic conditions.

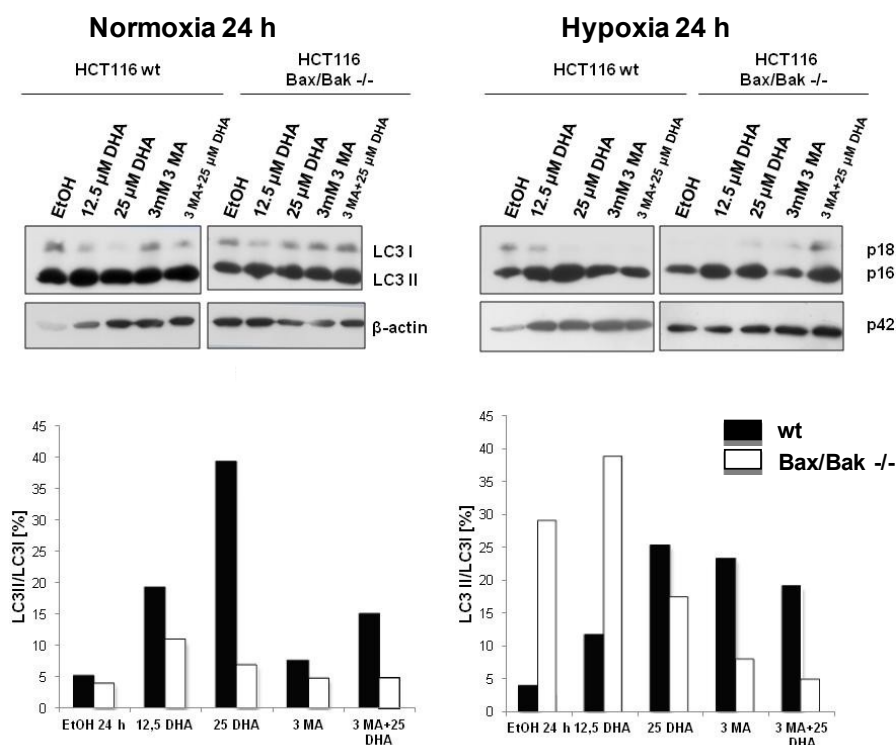


**Fig. 38 DHA induces autophagy in Bax/Bak independent manner in normoxia and hypoxia.** HCT116 wt and HCT116 Duo Bax/Bak neg. cells were treated with DHA (0-25  $\mu$ M DHA) and 12 h later were analyzed by immunofluorescence, using a specific antibody which binds to LC3. The experiment was performed two times and the representative pictures were used. Scale bar corresponds to 10  $\mu$ m.

By Western blot analysis of the LC3II proteins, it was confirmed that autophagy was induced at basic levels, approximately 5 %, in HCT116 wt cells under both normoxic and hypoxic conditions (Fig. 39). DHA induced significant amount of autophagy in dose dependent manner leading to maximum amount of autophagy to 40 % in HCT116 wt in normoxia. DHA induced also significant increase in autophagy in hypoxia, however it was lower (25 %) in comparison to normoxia. In contrast to the HCT116 wt, in



HCT116 Bax/Bak negative cells, autophagy was lower in normoxia (10 % at 12.5  $\mu$ M DHA) but in hypoxia it was highly up-regulated (40 % at 12.5  $\mu$ M DHA).



**Fig. 39 Autophagy is Bax/Bak-dependent in normoxia but not in hypoxia (A)** HCT116 wt and HCT116 Bax/Bak -/- cells were treated with DHA in normoxia and hypoxia and 24 h later the cells were harvested and the whole cell lysates were prepared. For autophagy characterization, a specific antibody against LC3 was used recognizing LC3I and LC3II form. The experiment was performed three times and the representative blots are shown. **(B)** Graphs represent densitometric analysis from the current blot.

### 3.2 Combination treatment with ionizing radiation

Hypoxic tumor microenvironment leads to limited success of radiotherapy. Molecular  $O_2$  must be present during the radiation exposure in order to fix the DNA damage and produce free radicals (Hall and Giaccia 2006). As Dihydroartemisinin (DHA) is radical-forming endoperoxide, the idea was to overcome therapy resistance of tumor cells under acute hypoxia by using this drug for combination treatments with ionizing radiation.

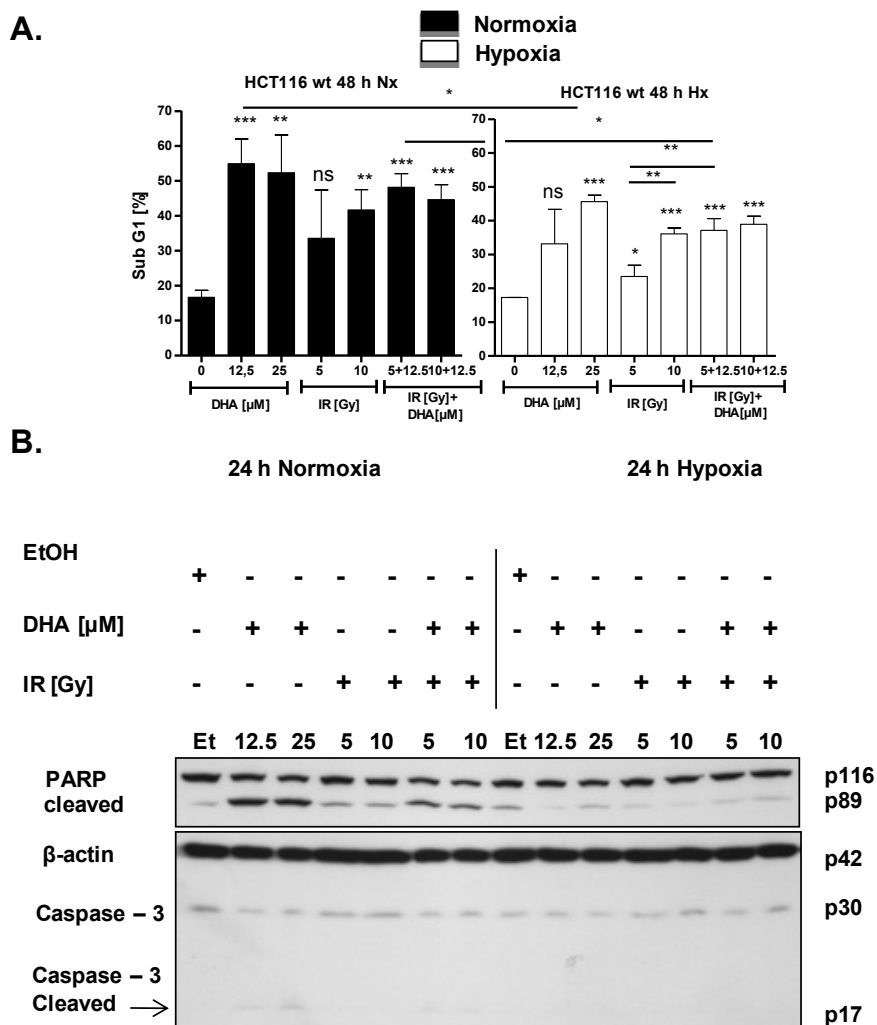
### 3.2.1 DHA in combination with ionizing radiation in HCT116 model

In a first part, it was tested whether DHA in combination with radiotherapy was synergistically inducing apoptosis in solid colon cancer cells in normoxic and hypoxic conditions.

For the combination experiments, HCT116 wt cells were used and treated with DHA, ionizing radiation or the combination of both treatments. In case of combination, DHA was added 10 minutes before the radiation. Apoptosis was studied by Western blot or FACS analysis. To elucidate the efficacy of combined treatment under hypoxia, the experiments were done under both normoxic and hypoxic conditions.

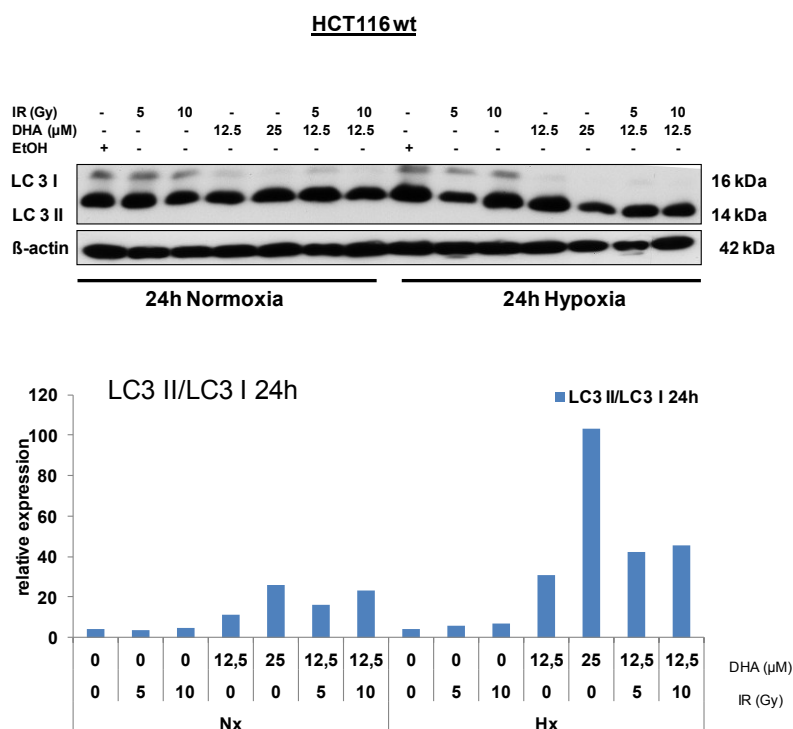
FACS analysis after 48 h revealed that DHA alone induced significant apoptosis in HCT116 wt cells under normoxic and hypoxic conditions, as it was already described in 3.1.5 (Fig. 40). Ionizing radiation was able to induce a high amount of apoptosis of HCT116 wt cells under normoxic conditions (45 % cells in Sub G1 in response to 10 Gy) and under hypoxic conditions (35 % cells in Sub G1 in response to 10 Gy) but it was lower in comparison to DHA alone (50 % in normoxia and 45 % in hypoxia cells in Sub G1). In combined treatments of DHA and irradiation, apoptosis induction was exactly like DHA alone under normoxic conditions. However, under hypoxic conditions, apoptosis was significantly increased in combined treatment in comparison to irradiation alone but not to the drug alone. DHA alone was more prominent in apoptosis induction than ionizing radiation. On the one hand apoptosis was significantly reduced under hypoxic conditions in case of low DHA concentration or low irradiation dose (not significantly) in comparison to normoxia. On the other hand, higher dose of both treatments (25  $\mu$ M DHA and 10 Gy) were sufficient to induce similar amount of apoptosis under both conditions (Fig. 40 A). To confirm the results from FACS data, caspases activation was further investigated by Western blot analysis. Caspase-3 activation was determined 24 h after treatments with DHA, ionizing radiation and the combination of both treatments. DHA alone showed high amount of PARP cleavage which is a caspase-3 substrate in normoxia. Interestingly, ionizing radiation alone achieved small amount of apoptosis induction. However, in combination treatments, PARP cleavage was significantly increased in comparison to the control or irradiation alone but not more than DHA alone in normoxia. Caspase-3 cleavage was hardly detectable in DHA treated cells and in combined treated cells with irradiation.

Caspase-3 activation was abolished under hypoxic conditions, in all the treatments (Fig. 40 B).



**Fig. 40 Combination treatment induces non synergistic apoptosis in HCT116 wt cells.** HCT116 wt cells were treated with DHA, ionizing radiation or with combination of both. **(A)** A FACS analysis was performed at 48 h time point of treatments in normoxia and hypoxia. The graphs represent means  $\pm$ SD from triplicate experiments. Statistical analysis was performed according to unpaired two tailed t-test (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ . Experiments were performed in triplicate. The (\*) without associated lines, represent the significant difference in comparison to the ethanol control. **(B)** Whole cellular extracts were prepared after 24 h of incubation under normoxic or hypoxic conditions. The experiment was performed in triplicate and representative blot was used.

However, in combination treatments, PARP cleavage was significantly increased in comparison to the control or irradiation alone but not more than DHA alone in normoxia. Caspase-3 cleavage was hardly detectable in DHA treated cells and in combined treated cells with irradiation. Caspase-3 activation was abolished under hypoxic conditions, in all the treatments (Fig. 40 B).



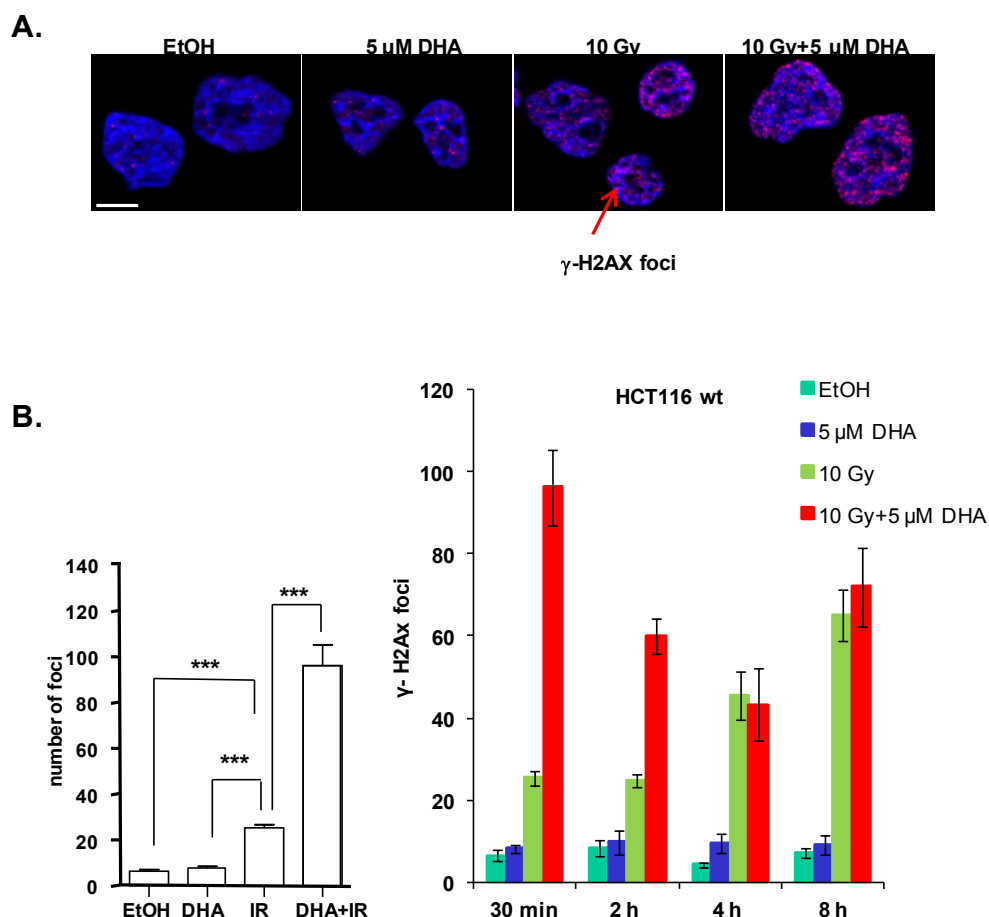
**Fig. 41 DHA-induced autophagy is stronger in hypoxia and in combination treatments.** HCT116 wt cells were plated for whole cell lysates and treated under normoxic or hypoxic conditions. At 24 h time point, cells were harvested and whole cell lysates were prepared. The experiment was performed in duplicate. The densitometric analysis was made from representative blot by Photoshop

As shown from previous results, apoptosis was not increased in combination treatments more than in DHA alone. Therefore, the question remains, whether the combination treatment could induce autophagy. To analyze autophagy in combination treatments with ionizing radiation, whole cell lysates were prepared from HCT116 wt cells under normoxic and hypoxic conditions. LC3 cleavage was analyzed by Western blots and the ratio of LC3II (associated with autophagosomes) to LC3I (associated with cytoplasm), was calculated and considered as indicative for autophagy induction. Preliminary results from the densitometric analysis showed that DHA-induced autophagy was upregulated under normoxic and hypoxic conditions but autophagy was much higher in hypoxia especially at 25 μM DHA. In combination treatments, autophagy was further increased compared to the single treatments (Fig. 41).

### 3.2.2 Combination treatment induced persistence of DNA double strand breaks (DBS)

As described in 1.2.7, the ionizing radiation induces cellular stress and DNA damage. DNA double-strand breaks belong to the most serious lesions and correlate with cell killing. At this point it was interesting to test whether DHA induced DNA damage and whether DNA damage could be further increased in combination treatments with ionizing radiation.

For these experiments HCT116 wt cells were used. The cells were treated with 5  $\mu$ M DHA and the dose of 10 Gy of ionizing radiation was applied. For combination treatments (DHA + irradiation), DHA was added 10 minutes before the irradiation.



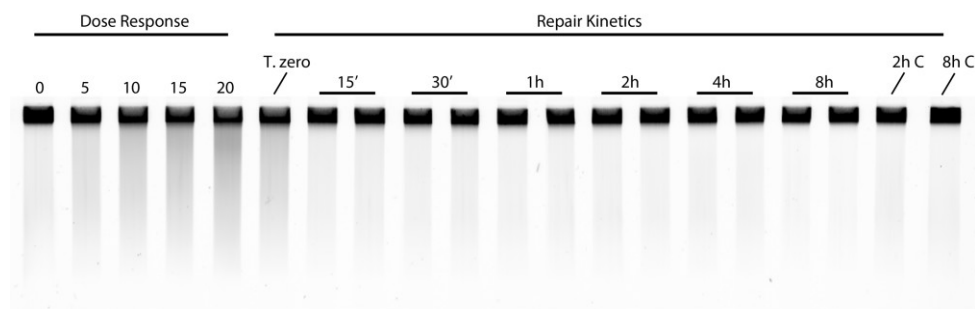
**Fig. 42 Combination treatments induced DNA damage persistence.** (A) HCT116 wt cells were treated with DHA or ionizing radiation or the combination of both. The cells were analyzed by immunofluorescence and representative pictures from 30 minutes time point are shown. (B) The  $\gamma$ -H2AX foci were quantified and represented on graph  $\pm$  standard error. At least 3 pictures were analyzed and quantified. The results are from 2 experiments and the significance test was performed from the means and standard errors from number of  $\gamma$ -H2AX foci per approximately 100 nuclei. Statistical analysis was performed according to unpaired two-tailed *t*-test (\*\*\*)  $P < 0.001$ .

Repair kinetics was monitored by immunofluorescence detecting a specific marker for DNA double strand breaks, the phospho H2AX ( $\gamma$ -H2AX). Usually DNA damage is repaired within two hours after radiation (Wierowski, Thomas et al. 1984). The results from immunofluorescence showed that DHA induced a minimal DNA damage which was not significant in contrast to the ethanol solvent control and it was not influenced over the time. In case of ionizing radiation alone, the DNA damage was significantly increased over the time. In combination treatments the  $\gamma$ -H2AX foci were rapidly increased at 30 minutes time point (100  $\gamma$ -H2AX foci) (Fig. 40 A), however, the number of cells with DNA double strand breaks were decreased over the time until 4 hours time point and up-regulated again after 8 hours (Fig. 40 B). Irradiation induced significantly less  $\gamma$ -H2AX foci at early time points until time point 4 h in comparison to the combination treatment and from this time point (4 h) combination treatments showed to induce the same number of  $\gamma$ -H2AX foci until 8 h time point.

To confirm the results from immunofluorescence, the same samples were also analyzed by pulsed field gel electrophoresis (PFGE). This method is widely used to detect the induction and repair of double-strand breaks.

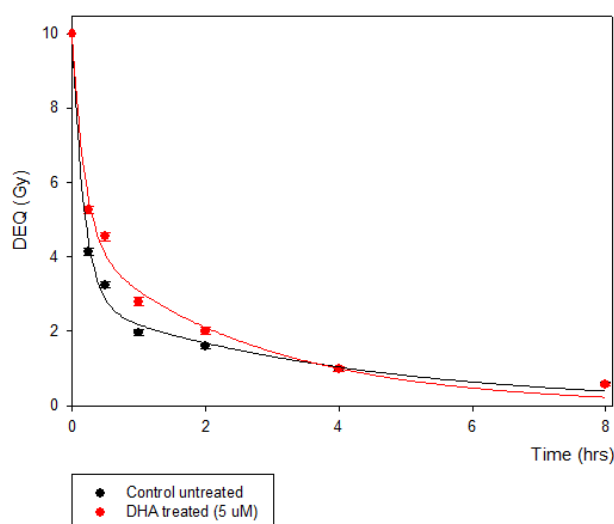
The results showed a delay in repair kinetics when the cells were treated in combination with DHA and ionizing radiation in comparison to radiation alone until the time point 4 h (Fig 43).

A.



B.

Repair Kinetics HCT116 cells exposed to a 10 Gy dose (X-rays)



**Fig. 43 Combination treatment with ionizing radiation shows delay in repair kinetics at early time points.** From HCT116 wt cells in Fig. 42, agarose plugs within which cells have been embedded and lysed were runned by PFGE for 40 hours. The fraction of DNA released (FDR) from the agarose plug was quantified for each time point with ImageQuant 5.2 (GE-Healthcare). The experiment was performed two times. The DNA in gel plugs were loaded in 3 different gels (3 identical replicates) in each experiment.

### 3.2.3 Combination treatment leads to increase of clonogenic death in HCT116 wt cells

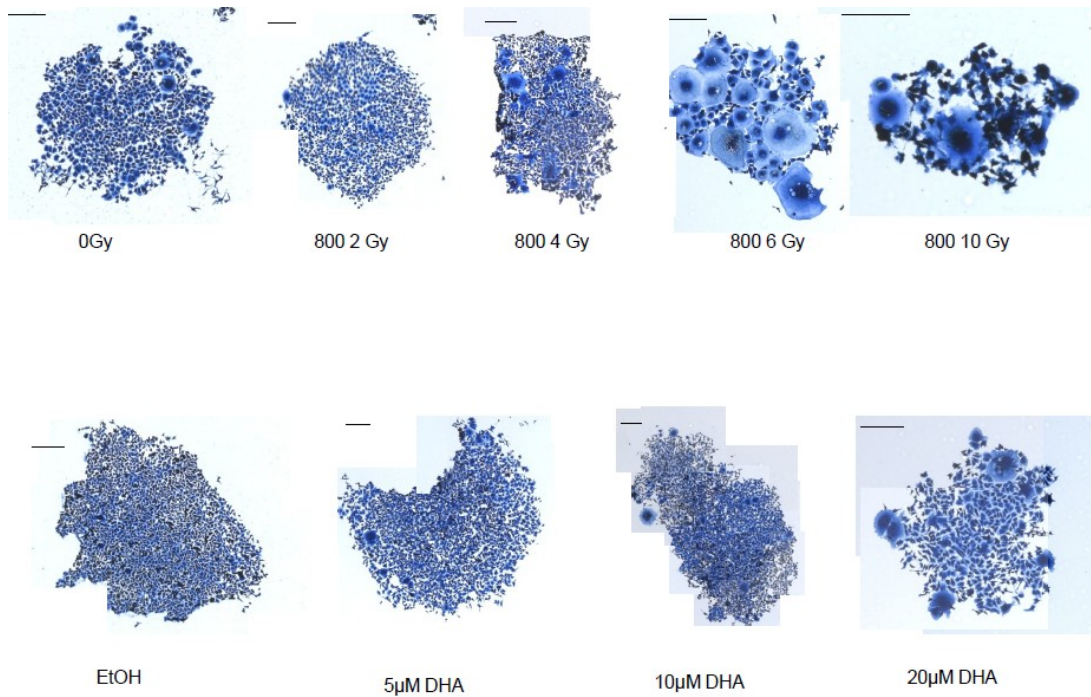
The next aim was to investigate the antineoplastic efficacy of DHA in combination treatment in HCT116 wt focusing on long term assays. For this purpose, HCT116 wt cells were plated for colony formation assay. The cells were treated with different DHA concentrations or different doses of ionizing radiation or the combination of both treatments. The experiments were

performed in normoxia according to the protocol of delayed plating (described in 2.2.11).

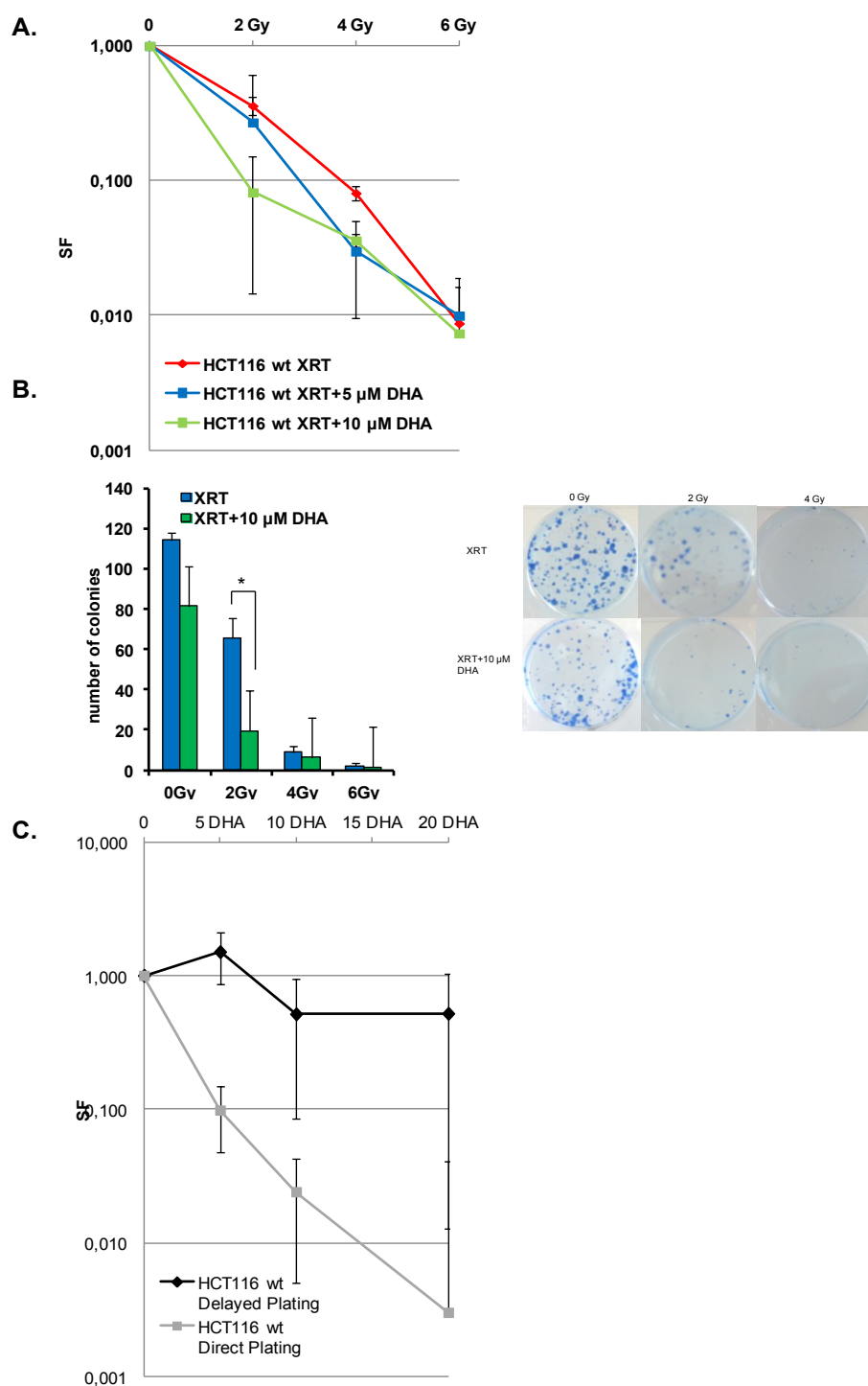
At the end of the experiment, the colonies were visualized under the microscope and studied to evaluate the number as well as the shape of the colonies. As shown in the Fig. 44, the cells which were irradiated with 4-6 Gy showed a big swollen cytoplasm and “giant” nuclei. This phenotype was not seen in cells treated with 0-10  $\mu$ M DHA alone. However, higher dose of 20  $\mu$ M DHA and the combination treatments also showed an increase in the number of “giant” cells.

The colonies were quantified and normalized to their ethanol controls. The calculated survival fractions are presented in fig. 45 A. Ionizing radiation (4-6 Gy) led to a significant eradication of colonies, showing sensitivity in those cells to ionizing radiation. On the other hand when cells were treated in combination with DHA, the number of colonies was reduced even more than when ionizing radiation alone was applied (Fig. 45 B). Interestingly, DHA presence influenced HCT116 wt cells response to the treatment. In more details, when HCT116 wt cells were plated directly for the colony formation assay and DHA was present all the time during the experiment, the survival fraction was decreased in dose dependent manner. In contrast, when the cells were plated, treated with DHA and 24 h later re-plated for the colony formation assay (delayed plating), they were able to recover and the survival fraction was significantly higher compared to the survival fraction from the direct plated cells (Fig. 45 C).





**Fig. 44 Ionizing radiation in combination with DHA leads to an altered cell phenotype.** HCT116 wt cells were plated for colony formation assay according to a protocol for delayed plating described (2.2.11.2). 800 cells were plated in 6 well and after 10 days the experiment was stopped and the colonies were fixed and stained with coomassie blue. The colonies were analyzed under the microscope and the pictures are the representative from two independent experiments. Each colony represents a combination of 3 or more pictures together. The scale bar corresponds to 100 μm.



**Fig. 45 Combination treatment of ionizing radiation and with DHA leads to eradication of clonogenic HCT116 wt cells.** (A) HCT116 wt cells were plated for colony formation assay with delayed plating. 200 – 64000 cells were plated and incubated for 10 days until the colonies were grown. The survival fraction was calculated after counting the number of colonies and normalized by plating efficiency. Survival fractions were represented from two independent experiments. (B) Colonies were quantified and displayed on a graph from correspondent plates with 800 cells plated. Statistical analysis was performed according to unpaired two-tailed *t*-test (\*\*)  $P < 0.01$ ; (\*\*\*)  $P < 0.001$ . (\*) corresponds to XRT (C) The Survival fraction from direct plating from fig. 37 was compared to the survival fraction from the delayed plating in DHA treated HCT116 wt cells.

## 4 Discussion

### 4.1 DHA Treatment

#### 4.1.1 DHA induces growth inhibition and apoptosis induction under normoxic and hypoxic conditions

This study showed that DHA induced inhibition of cell proliferation and apoptosis in dose and time dependent manner in human colon cancer cells (HCT15, Colo205 and HCT16), in Jurkat T-lymphoma cells and in prostate cancer cells (Du145). These data corroborate findings obtained by other groups in distinct cells, as in a study on human hepatocellular carcinoma cells (HCC) (Hou, Wang et al. 2008) and HUVECs cells (Chen, Zhou et al. 2003). Most of the studies on artemisinin derivatives activity have been performed under normoxic conditions and little is known about artemisinin derivatives activity under severe hypoxia. The current study showed for the first time, that ROS-donor DHA was active also in severe hypoxia at 0.2 % of O<sub>2</sub>, although higher doses of DHA were necessary to achieve significant apoptosis. Interestingly, a recent study in human dermal microvascular endothelial cells (HMEC-1) demonstrated that DHA induced apoptosis was decreased at 1% O<sub>2</sub>. However, DHA activated caspases (caspase-3 and 7) under both normoxic and hypoxic conditions and the caspase activation was stronger under hypoxic conditions (D'Alessandro, Basilico et al. 2011). These observations are in contrast to the current study as significantly lower activation of caspase-3 and inactivation of its substrate PARP in comparison to the normoxic conditions was observed. That comes to a conclusion that DHA induced antineoplastic effect under both conditions, however in normoxia the cells preferentially died by apoptosis while, under hypoxic conditions, other mechanism was induced which was caspases independent. On the other hand viability assays as WST-1 and trypan Blue showed a strong cytotoxic and cytostatic effect under normoxic conditions in dose-dependent manner, however, in hypoxic conditions DHA induced mostly cytostatic effect and cytotoxicity was observed only at higher DHA concentrations in HCT116 wt cells.

It has been shown from our previous work and from other groups, that artemisinin derivatives induce their cytotoxic effect via ROS formation (Handrick, Ontikatz et al. 2010; Berdelle, Nikolova et al. 2011; Cabello, Lamore et al. 2011). This makes it even more challenging for the studies

under hypoxic conditions where artemisinin derivatives could be combined with the classical chemotherapy, such as radiotherapy. In this case, artemisinin derivative DHA would act as a donor of ROS and improve the outcome of therapy in resistant hypoxic tumors.

#### **4.1.2 DHA induced necrosis in normoxia and hypoxia**

DHA induced not only apoptosis but also necrosis in colon cancer (HC15, colo205 and HCT116), Jurkat A3 and in prostate cancer (Du145) cells. Interestingly, in normoxia DHA-induced necrosis was increased in Jurkat A3 cells when pan-caspase inhibitor zVAD was applied in combination with DHA. This indicates that DHA can induce apoptosis independent death mode by necrosis. Moreover, DHA induced necrosis in a dose and time dependent manner in normoxia, however, it was not further increasing over the time in hypoxia according to Hoechst and PI staining in colon cancer cells (HC15, colo205 and HCT116). Thus, DHA induced necrosis was stronger under normoxic conditions than under hypoxic conditions in HCT116 wt. In contrast to HCT116 wt cells, Colo205 showed only 10 % of necrotic cells in response to high DHA concentration (50  $\mu$ M) and this number increased to 50 % at the same DHA concentration in hypoxia. Necrosis was significantly increased in HCT15 cells in both normoxia and hypoxia in response to high DHA concentrations (25-50  $\mu$ M). Those results revealed that DHA induced apoptosis in both normoxic and hypoxic condition, however necrosis is induced with different mode, dependent from the cell line. In a similar study on Chinese hamster ovary cell line, necrosis induction was shown according to annexin V/PI staining in artesunate treated cells at 72 h time point. Necrosis was increasing in a dose dependent manner reaching 32 % of necrotic cells in response to 50  $\mu$ g/ml of artesunate (Li, Lam et al. 2008; Berdelle, Nikolova et al. 2011).

Here, it was for first time shown that DHA could induce necrosis in hypoxia. At lower concentrations of DHA (10-25  $\mu$ M), apoptosis was preferentially induced in all the discussed cell lines, however, in response to higher DHA concentration (50  $\mu$ M), death by necrosis was more prominent. This is a very interesting result as it shows that DHA can induce alternative death pathways in case of apoptosis defective tumor cells.

### 4.1.3 DHA-induced apoptosis and cell cycle arrest is Bax/Bak dependent under both, normoxic and hypoxic conditions

The importance of Bcl-2 family associated to DHA action was studied focusing on the pro-apoptotic proteins Bax and Bak. In our previous studies on JCAM Bak negative and Bak positive cell lines, it was shown that DHA induced caspases activation and apoptosis in Bak dependent manner. DHA downregulated the anti-apoptotic protein Mcl-1 and upregulated the pro-apoptotic protein Noxa in JCAM Bak positive cell line, but not in Bak negative cells (Handrick, Ontikatzte et al. 2010).

In the current study, Bax/Bak positive or deficient tumor cells (HCT116 wt, Bax negative, Bak negative and Bax/Bak negative as well as Du145 mock or Du145 Bax) were used. Results revealed that DHA was able to induce apoptosis in Bax/Bak dependent manner under normoxic and hypoxic conditions. In our colon cancer model it was observed that loss of Bax could not be substituted in its role in apoptosis induction by Bak, however, Bak negative cells could still undergo to a significant induction of apoptosis and Bax was sufficient to induce a similar amount of apoptosis as was the case in HCT116 wt cells which are Bax and Bak positive. In this model it can be concluded that DHA induced apoptosis in HCT116 in Bax dependent manner but not Bak, as was shown in JCAM Bak positive cells. Bax importance in apoptosis induction was also confirmed from a similar study using the same HCT116 model but other chemotherapy (Chandra, Choy et al. 2005; Wang and Youle 2011). The importance of Bax could be explained in HCT116 cells in consideration of Mcl-1 inhibition of Bak in the absence of Bax in response to classical chemotherapy (Gillissen, Wendt et al. 2010; Wang and Youle 2011). In this case with the silencing of Mcl-1 should be possible to induce apoptosis also in Bax absence. Indeed, in a recent study the silencing of Mcl-1 by siRNA sensitized Bax negative cells to chemotherapy-induced apoptosis according to caspases activation and cytochrome-c release. Contemporary, the overexpression of Noxa was also sufficient to induce apoptosis in Bax negative cells (Wang and Youle 2011). Interestingly, DHA induced Mcl-1 downregulation and Noxa upregulation and that was sufficient to activate Bak and induce apoptosis in dose dependent manner in JCAM Bak positive cells (Handrick, Ontikatzte et al. 2010). However, DHA did not downregulate Mcl-1 protein levels in HCT116 wt and HCT116 Bax/Bak negative cells in both normoxic and hypoxic conditions (Fig. S1).

In Du145 prostate cancer cells, Bax expression significantly supported DHA-induced depolarization of the mitochondrial membrane potential and DNA fragmentation. In contrast to the Du145 Bax positive cells, Du145 mock cells, which were only Bak positive but Bax negative, showed no depolarization of the mitochondrial membrane potential or DNA fragmentation in response to DHA. Taken together these results showed that in HCT116 and Du145 model, Bax and not Bak, plays a pivotal role in DHA-induced apoptosis. By contrast, loss of Bax in Du145 induced significant necrosis in response to DHA.

Bax activation showed to have a pivotal role for DHA action under both normoxia and hypoxia in HCT116 wt cells as it was shown by FACS analyses with Bax translocation in the nucleus.

Under hypoxic conditions, HIF-1 and p53 interact in many ways to finally rule for the cell fate. Hypoxia is known to protect cells from apoptosis induction in response to classical chemotherapy such as etoposide. It was reported that targeting HIF-1 could significantly enhance apoptosis in hypoxia (Sermeus, Cosse et al. 2008). In the current study it was shown that hypoxia itself did not induce apoptosis in HCT116 model, however, DHA induced significant amount of apoptosis with regard to the FACS results ( $\Delta\psi_m$  and SubG1) in acute hypoxia 0.2% O<sub>2</sub>. Worthy of mention, apoptosis was strongly downregulated in absence of Bax. In addition, in other colon cancer cells, such as colo205 and HCT15, DHA showed to induce both apoptosis and necrosis under both normoxic and hypoxic conditions.

Taking together the data from all the described models, it can be concluded that DHA acts in Bax Bak dependent manner (HCT116, Du145 and JCAM), but with specific differences dependent on cell type and the microenvironment. Bax plays an important role in HCT116 and Du145 model, leading however to a different apoptosis induction. Du145 cells are p53 negative and this could explain the apoptosis decrease in comparison to the HCT116 cells which are p53 wt. JCAM Bak positive cells, lacking both p53 and Bax, were sensitive to apoptosis induction by DHA. In this last model, Bak was sufficient to induce apoptosis in response to DHA via Mcl-1 downregulation and Noxa upregulation. DHA may preferentially depend from Bax or Bak for the apoptosis execution in different cell models described.

The importance of Bax for apoptosis induction was also described in previous studies on treated ovarian cancer cells. In particular, DHA was shown to induce an increase in Bax protein level according to the Western blots analysis, as well as to the mRNA level measured by RT-PCR, in p53 independent manner. Loss of Bax by siRNA almost abolished apoptosis induction in ovarian cancer cells in normoxia (Jiao, Ge et al. 2007). This favors the data obtained in the current study, in which is shown that DHA induced apoptosis is Bax dependent in normoxic conditions. It is necessary to mention that it has never been described before that in HCT116 model DHA induces apoptosis under normoxic and, at a lower amount, under hypoxic conditions in a Bax dependent manner.

Focusing on cell cycle distribution in response to DHA, HCT116 wt and HCT116 Bax/Bak negative cells were analyzed under normoxic and hypoxic conditions. According to the FACS data, DHA induced G2 arrest in normoxia and G1 arrest in hypoxia, however in the both cases the difference was not significant. On the other hand loss of Bax/Bak did not influence the cell cycle distribution in response to DHA in G1 and G2 phases under both normoxic and hypoxic conditions in comparison to the ethanol controls. Interestingly, S phase significantly decreased under hypoxic conditions in HCT116 Bax/Bak negative cells. In similar studies from other groups, in ovarian cancer cells and human osteosarcoma cells showed significant increase in G2 and decrease in G1 phase in normoxic conditions (Jiao, Ge et al. 2007; Ji, Zhang et al. 2011). On the other hand DHA induced cell cycle arrest in G1 phase in HCT116 colon cancer cells and pancreatic cancer BxPC- cells in normoxia (Chen, Sun et al. 2010; Lu, Chen et al. 2011). In addition, DHA induced G2 arrest in immortalized human microvascular endothelial HMEC-1 cells at dose (12.5 -50  $\mu$ M DHA) in both normoxic and hypoxic conditions (D'Alessandro, Basilico et al. 2011). Those results show that DHA-induced cell cycle arrest depending on cell type. These inconsistencies are probably due to metabolic features of the cells leading to different growth rates. The G2 cell cycle arrest could be caused by DNA damage (Li, Lam et al. 2008).

It has been reported that DHA exerts cytotoxic effects by inhibiting the hypoxia inducible factor and its targets, such as VEGF. DHA exerted selectively on glioma C6 cells by increasing the reactive oxygen species and inhibited hypoxia inducible factor HIF-1 $\alpha$  as well as its target VEGF in dose and time dependent manner (Huang, Ma et al. 2007). Here was

examined HIF-1 $\alpha$  expression at the protein level in HCT116 wt, HCT116 Bak  $-/-$ , HCT116 Bax  $-/-$  and HCT116 Bax/Bak  $-/-$  over the time (2 h-24 h Hypoxia).

Results revealed that HIF-1 $\alpha$  expression started to be detectable at 2 h time point and it was increased over the time until 12 h and decreased at 24 h time point in all the cell lines. In addition, from preliminary data, DHA influences the protein level of HIF-1 $\alpha$  in Bax/Bak dependent manner. HCT116 wt cells showed no differences in HIF-1 $\alpha$  expression in response to DHA treatment. Interestingly, HCT116 Bak  $-/-$ , HCT116 Bax  $-/-$  and HCT116 Bax/Bak  $-/-$  showed decrease in HIF-1 $\alpha$  levels to half in DHA treated cells in comparison to the ethanol controls at 12 h time point. In conclusion DHA induced downregulation of HIF-1 $\alpha$  and it was even stronger in Bax/Bak absence. That is an important observation because HIF-1 correlates with poor prognosis in classical chemotherapy (Unruh, Ressel et al. 2003; Brown, Cowen et al. 2006; Sullivan, Pare et al. 2008).

#### **4.1.4 DHA induces increase in autophagy**

In contrast to the short term experiments, long term experiments, such as colony formation assay, showed that, DHA induced clonogenic death in HCT116 cells independently of Bax or Bak presence under hypoxic conditions indicating for the alternative mechanism which is taking place in DHA-induced death.

Investigating the alternative pathways that could contribute to the Bax and Bak independent cytotoxic effect of DHA, autophagy was promising mechanisms of DHA-induced death. Based on data from immunofluorescence and western blots analyses, for the first time it was shown that DHA induced autophagy in HCT116 wt and HCT116 Bax/Bak deficient cells and autophagy was strongly increased under hypoxic conditions in HCT116 Bax/Bak negative cells. This may suggest an alternative mechanism involved in DHA induced Bax/Bak independent clonogenic death.

From studies in Jurkat A3 cells, in which anti-apoptotic proteins such as Bcl-2 were overexpressing, Helanin, a sesquiterpene lactone, could bypass Bcl-2 mediated cell death resistance. ROS formation was required for cell death and the key factor found to be involved in Bcl-2 overexpressed Jurkat cells death was the Nf-kB inhibition. In contrast to the current study, they could not observe autophagy induction (Hoffmann, von Schwarzenberg et al. 2011). Here was also shown that DHA induced autophagy not in all cell



models. In prostate cancer Du145 mock and Bax positive DHA treatment showed no autophagy induction. This could be explained because Du145 cells are p53 negative and p53 plays an important role in positive regulation of autophagy by activating specific targets such as DRAM family (D'Amelio and Cecconi 2009; Maiuri, Malik et al. 2009; Ryan 2011). From another study, deficiency of apoptotic effectors Bax and Bak, showed to induce autophagic cell death pathway in response to chemotherapy (Dolmans, Fukumura et al. 2003).

Most of the studies on artemisinin derivatives have been done in normoxia. However, little is known about their action in hypoxia. The present study introduces new explanations of DHA action under hypoxic conditions. It may be that DHA acts through autophagy induction in response to Bax/Bak absence in HCT116 model and, in case of prostate cancer cells which are not undergoing autophagy, DHA treatment induces an increase in necrosis in Bax absence. The other important observation is that DHA is able to induce apoptosis and necrosis in normoxia and hypoxia in p53 independent manner as shown from results in HCT15 (p53 mutated cells) and colo205 with p53 wt. In addition, DHA induced a small amount of apoptosis also in Bax/Bak negative cells, thus suggesting that DHA can induce apoptosis by activation of alternative apoptotic pathway, independently from the intrinsic apoptotic pathway. Finally, DHA decreased HIF-1 $\alpha$  protein levels which suggests further contribution in the antineoplastic efficacy of clonogenic Bax/Bak deficient tumor cells.

## 4.2 DHA in combination treatments

The second aim of this work was to study whether DHA acts as a sensitizer to ionizing radiation treatment in normoxia and hypoxia in HCT116 wt cells. Several studies have been done using the artemisinin derivative in combination treatments with Gemcitabine (Hou, Wang et al. 2008), TRAIL antibodies (Handrick, Ontikatz et al. 2010), ionizing radiation (Kim, Kim et al. 2006) and other chemotherapeutics (Zhou, Zhang et al. 2010). In all those studies was shown that artemisinin derivatives acted as sensitizers to chemotherapy.

In the current study, it was first investigated whether apoptosis induction was increased in combination treatments in HCT116 wt cells. It was observed by FACS and Western blot analyses that DHA did not additionally contribute in apoptosis induction in combination treatments in comparison to the single treatments under both normoxic and hypoxic conditions. In

JCAM Bak positive cells it has been shown that DHA synergistically induced apoptosis in combination with radiation treatment (Handrick, Ontikatzte et al. 2010), suggesting different responses in different tumor models.

As it was previously described, DHA produces reactive oxygen species (Berman and Adams 1997; Handrick, Ontikatzte et al. 2010) and it is essential for its cytotoxic effect. ROS importance in autophagy induction was studied by Jae-Min Yuk and collaborators. They focused on chemotherapy of colon cancer with cell wall skeleton of *mycobacterium bivia* Bacillus Calmette-Guerin (BCG/CWS). They found that when it was applied in combination with ionizing radiation in colon cancer treatment resulted in the induction of caspase independent death by autophagy. This result was achieved by the generation of ROS (Yuk, Shin et al. 2010). This work suggests that generation of ROS could lead to a cell death by autophagy. It could give a hint that ROS formations could sensitize the cells to ionizing radiation by inducing autophagic death. Indeed, DHA showed additive effect on the induction of autophagy in comparison to the single treatments with even stronger effect in hypoxia. The results showed that DHA differentially acts as a sensitizer to ionizing radiation depending on the tumor model.

Focusing further on the mechanism of DHA action in combination with ionizing radiation, results revealed that DNA double strands breaks were not efficiently repaired over the time (in comparison to the only irradiated cells) until the time point 4 h. In contrast to Berdelle et al. , DHA did not induce DSBs in comparison to the ethanol control and was not altered over the time. In parallel to this experiment, immunofluorescence was performed with the same conditions and this allowed showing that in combination treatment there was a significant increase in number of DNA double strand breaks already at the early time point (30 minutes) and these were significantly higher than in cells treated only with radiation. DBSs were reduced in number until 4 h time point and after were increased again. Those differences in number of DBSs/cell could be explained as artemisinin derivatives were reported to induce oxidative stress according to the 8-oxoG and EthenoA markers. It was shown that artesunate (15 µg/ml) induced a significant increase of DNA double strand breaks over the time differently when compared to ionizing radiation (Berdelle, Nikolova et al. 2011). That could suggest that due to accumulations of the oxidative

damage from the drug could lead to persistence of DNA damage over the time when it is combined with ionizing radiation. In the current study it is shown for the first time that DHA in combination treatment with radiation lead to the accumulation of DNA damage and finally led to clonogenic death in colon cancer cells (HCT116 wt). Here DHA showed to be a sensitizer of the cells to the radiation-induced death by suspected accumulation of DNA damage that lead to death. Long term experiments, as colony formation assay, convinced that DHA induced clonogenic death when used in combination with ionizing radiation in comparison to the single treatments. Similar observations have been done on U273MG glioma cells, in which DHA in combination with ionizing radiation enhanced the radiosensitizing effect and was highly dependent from ROS formation (Kim, Kim et al. 2006). The analyzing of the shape and structure of the colonies under the microscope helped to reveal that, when treated with ionizing radiation alone or in combination with DHA, the cells were showing a giant phenotype. The giant cells were suspected to be senescent; however, the cells showed negative staining for senescence (Fig. S2).

In addition from the colony formation assays, it was shown that when DHA was all the time present into the media of the cells, showed to be more toxic to the cells and it was leading to eradication of HCT116 wt cells. However, in case of delayed plating there DHA was only 24 h present and then replaced with fresh medium drug free, the cells were able to enter again the mitosis and recover with almost no eradication of the tumor cells. The differences between direct and delayed plating could be explained as in case of direct plating, it gives primary cytostatic effect from the drug as well as cytotoxic effect. In case of delayed plating it gives the information about the first kill (as long as the drug is present). From current study it comes clear that in single treatments with only DHA, the drug should be present in order to induce its cytotoxic effect for all the time period of the experiment. In contrast to current study, similar study on artesunate where the replacement of the drug by fresh medium showed to lead to higher apoptosis induction if the drug would be all the time present during the experiment (Liu, Gravett et al. 2011). On the other hand in single treatment with irradiation or in combination treatments showed to induce a significant eradication of the tumor cells using the delayed plating method. That suggests that DHA sensitizes tumor cells to ionizing radiation at early time point.

From the preliminary *in vivo* studies on human HCT116 xenograft model, it was shown that DHA in combination with ionizing radiation induced tumor growth delay in comparison to the controls or to the single treatments (Fig. S3) (Ontikatz, Henke et al. unpublished data). Similar results were obtained on human non-small cell lung cancer cells A549 applying combination treatments with artesunate and ionizing radiation (Zhao, Jiang et al. 2011). However, a stronger delay on tumor growth could not be seen when artesunate was applied instead of DHA in combination treatments in comparison to radiation alone (data not shown).

In summary, DHA enhanced the radiosensitivity of HCT116 colon cancer cells via accumulation of oxidative stress and DNA damage and possibly autophagy induction. It is hypothesized that ROS formation derived from DHA treatment could induce oxidative stress and DNA damage and thus, could help ionizing radiation to induce its cytotoxic effect.

### 4.3 Clinical relevance

As it was described in the introduction, artemisinin derivatives are widely used as anti malaria treatment in populations with high malaria incidence. The drug showed no side effects at concentrations in the nanomolar range. In addition, the drug showed selective toxicity to the parasites infected erythrocytes (Klayman 1985). Recently artemisinin derivatives showed to exert antineoplastic effects in cancer therapy *in vitro* and *in vivo* (Efferth, Dunstan et al. 2001; Dell'Eva, Pfeffer et al. 2004; Efferth 2005). Interestingly, in all the studies artemisinin derivatives showed selective activity only on tumor cells and were not affecting normal cells (Hou, Wang et al. 2008). This depends on the bio-activation of the drug, which requires iron, making the drug selectively active only on cells with high iron uptake. Highly proliferative cancer cells need high iron uptake and it has been reported that, in contrast to the normal cells, tumor cells express higher levels of transferrin receptor (Lai, Sasaki et al. 2005). In addition, a clinical case report on two patients with metastatic uveal melanoma presented preliminary evidence in support of the potential therapeutic benefit associated with artesunate chemotherapy (Berger, Dieckmann et al. 2005). The described observations make the artemisinin derivatives a good candidate for the tumor treatments with no side effects.

It is important to establish treatments that could sensitize solid tumor to classical chemotherapy and radiotherapy, considering that hypoxia is a

common phenomenon in solid tumors. In severe or prolonged hypoxia, tumor cells usually adapt to the environmental stress and escape apoptosis and necrosis during classical chemotherapy (Hickman, Potten et al. 1994). Here it was proposed to use ROS-donors, such as DHA, which showed to have an antineoplastic effect in acute hypoxia (0.2 % O<sub>2</sub>) and, moreover, it was able to sensitize tumor cells to ionizing radiation apoptosis and autophagy induction. Therefore, DHA is a promising candidate in hypoxic tumor therapy and in combination therapies with ionizing radiation.

#### **4.4 Proposed mechanism of DHA action under normoxic and hypoxic conditions**

It is hypothesized that DHA-induced apoptosis is due to ROS formation which is upstream of the mitochondria. It was shown from our previous work on Jurkat E6.1 cells that ROS scavengers, such as N-acetylcysteine or glutathione, were efficient to decrease significantly the ROS formation and, subsequently, to decrease apoptosis significantly induction (Handrick, Ontikatzte et al. 2010). The observation that ROS formation is required to induce apoptosis, making it even more interesting in hypoxia where ROS production would offer molecular oxygen and would help DHA to induce apoptosis under hypoxic conditions.

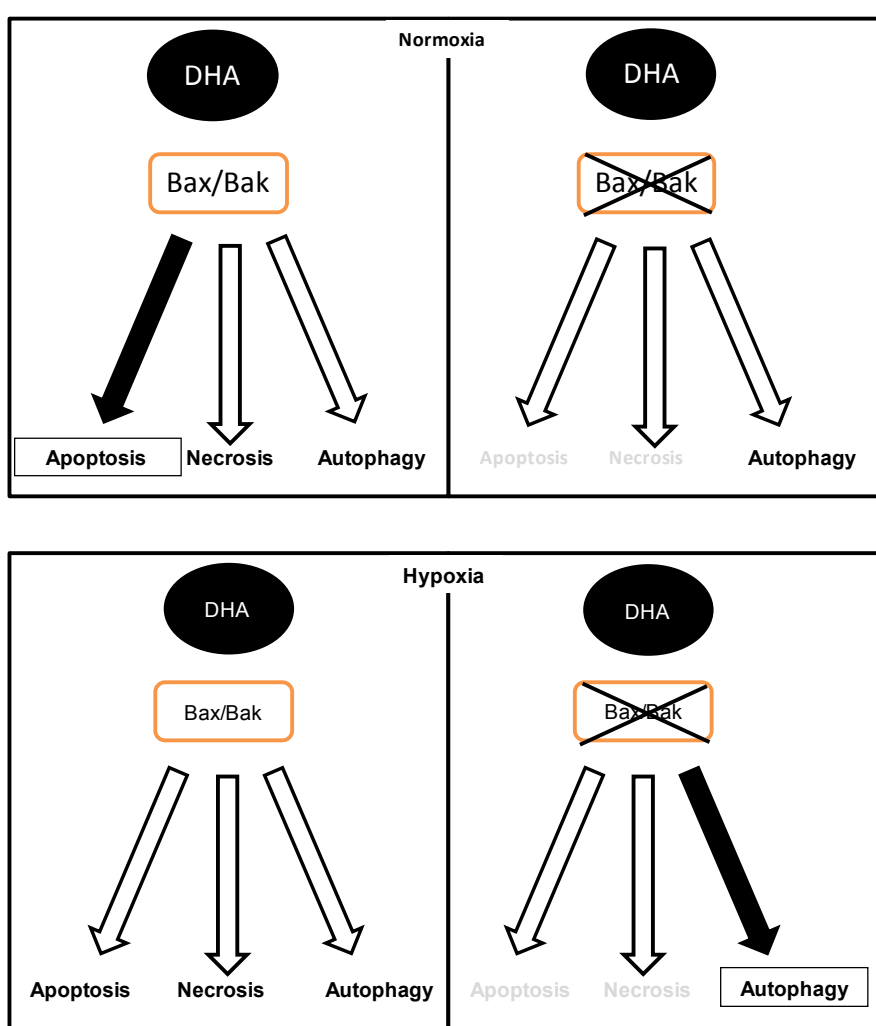
DHA-induced apoptosis was shown to be Bax dependent. Bax importance was concluded from Bax Bak positive HCT116 cells and Bax or Bak deficient cells. Loss of Bax had a significant abrogation in apoptosis induction and Bak was not able to substitute Bax in HCT116 model. When used on JCAM Bak positive T-Lymphoma cells, DHA showed to induce Bak dependent apoptosis via Mcl-1 downregulation and Noxa upregulation. Those results come to suggest that DHA induce apoptosis in Bax/Bak dependent manner depending from the cell type.

Another mechanism of death induced by DHA was necrosis. Necrosis was induced in all the described cell models. However, the exact mechanism behind DHA-induced necrosis is still not known. Interestingly, necrosis was enhanced when caspases were blocked by zVAD in Jurkat model and at higher DHA concentration (Fig. 46).

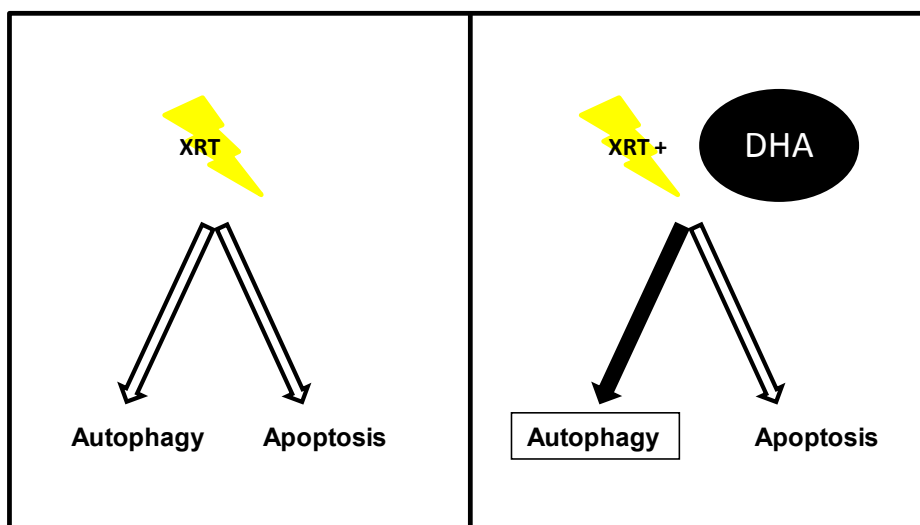
Finally, here was proposed autophagic death mode which was induced as an alternative death pathway in apoptosis deficient cells. DHA induced autophagy in HCT116 wt and HCT116 Bax/Bak negative cells and it was even higher under hypoxic conditions. Persistence of autophagy may

contribute to clonogenic death in Bax/Bak independent manner under hypoxic conditions.

In combination treatments with ionizing radiation, ROS formation induced significant apoptosis; however, apoptosis was not increased compared to single treatment with DHA under both normoxic and hypoxic condition. In addition, it was shown that in combination treatments autophagy was highly increased and showed additive effect under both normoxic and hypoxic conditions (Fig. 47). Interestingly, ROS formation induced persistence of DNA double strand breaks (DSBs) in combination treatments, however, DHA alone did not induce DSBs.



**Fig. 46 Suggested mechanism of DHA action in HCT116 model.** DHA induces apoptosis in a Bax/Bak dependent manner in HCT116 cells under normoxic and hypoxic conditions. Apoptosis is the most prominent death mode under normoxic conditions. In Bax/Bak deficient cells DHA induces autophagy, particularly under hypoxia. Therefore, It suggests that DHA induces autophagic death as an alternative death mode under hypoxic conditions and this may contribute to clonogenic death in Bax/Bak deficient cells.



**Fig. 47 Suggested mechanism in combination treatments in HCT116 model.**

The combination treatment of DHA with ionizing radiation showed increased autophagy under both normoxic and hypoxic conditions. In addition significant apoptosis was induced however it was not higher than DHA-induced apoptosis alone. Here, it is proposed that DHA-induced autophagy leads to XRT-induced clonogenic death in combination treatments.

## 5 Summary

The microenvironment of solid tumors is mostly characterized by regions of acute and chronic hypoxia which are known to decrease sensitivity of tumor cell to classical chemotherapy and radiotherapy. Here is proposed a novel strategy to overcome therapy resistance of solid tumor cells under acute hypoxia and to increase the efficacy of radiotherapy by using the radical-forming endoperoxide Dihydroartemisinin (DHA) that is known as anti-malaria drug and has only recently been shown to have antineoplastic activity. Aim of the current study was to evaluate the antineoplastic activity of DHA under normoxic and hypoxic conditions, to elucidate the importance of the intrinsic apoptotic pathway for drug activity in both conditions, and to study its potential to improve the cytotoxic action of ionizing radiation.

The cytotoxic effects of DHA (0-80  $\mu$ M) were evaluated in colon cancer cells (HCT15, Colo205 and HCT116) and Jurkat T-lymphoma cells using short term (apoptosis, necrosis, autophagy and viability assays) and long term assays (colony formation). HCT116 cells with or without expression of Bax, Bak, or both were used to study the importance of the intrinsic apoptotic pathway for efficacy of DHA in normoxia and hypoxia. For combined treatment, ionizing radiation (0-10 Gy) was applied 10 minutes after DHA treatment.

In normoxia, DHA efficiently induced concentration- and time-dependent apoptosis in Jurkat and colon cancer cells. The cells were also sensitive to DHA-induced apoptosis in hypoxia. Loss of proapoptotic Bax, Bak, or both largely decreased DHA-induced apoptosis in normoxia and hypoxia. Although loss of Bax, Bak or both impaired DHA-induced eradication of clonogenic tumor cells in normoxia, clonogenic cell death was almost not affected by loss of those proapoptotic proteins in hypoxia suggesting alternative death mechanisms. Autophagy was identified as an alternative cellular response in hypoxia. Cell death from autophagy may at least partially compensate for apoptosis deficiency in cells with defects of the intrinsic apoptosis pathway and may also contribute to the increased efficacy of radiotherapy in combination with DHA. Combination treatment with XRT increased clonogenic death in Bax/Bak positive HCT116 cells in normoxia and enhanced the persistence of  $\gamma$ -H2AX foci suggesting an impact of DHA on the repair of DNA double strands breaks or the accumulation of DNA damage by DHA-triggered oxidative stress.



All together findings of the current work suggest that DHA may be a good candidate for the treatment of hypoxic human tumors characterized by apoptosis resistance and for combined treatment with ionizing radiation.

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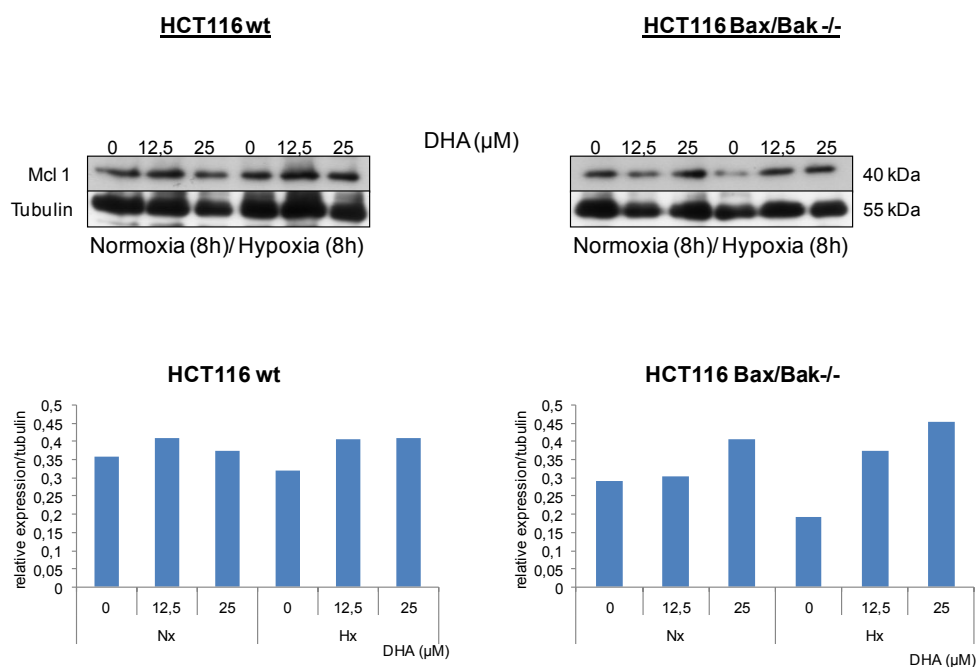


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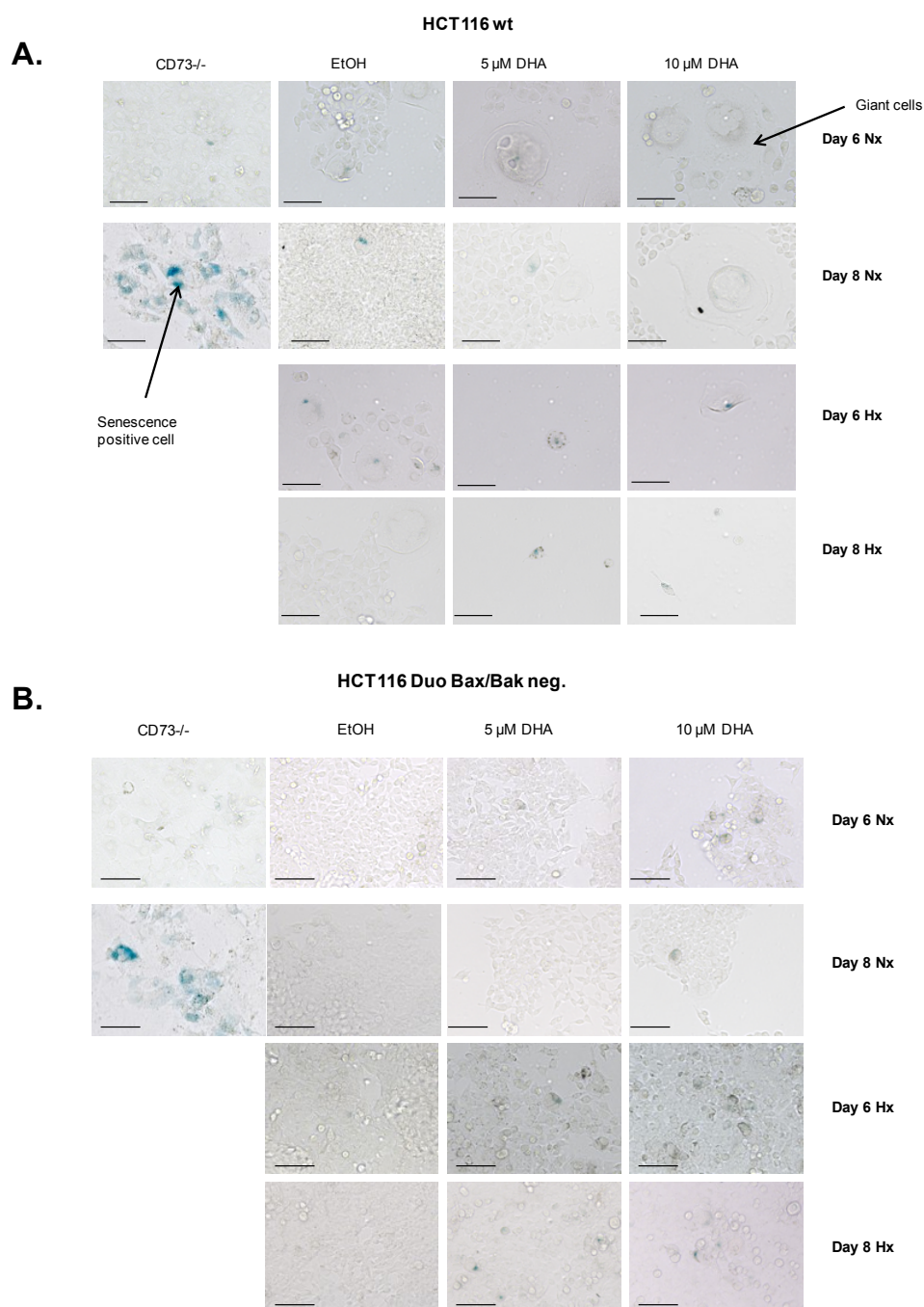
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## 7 Appendix

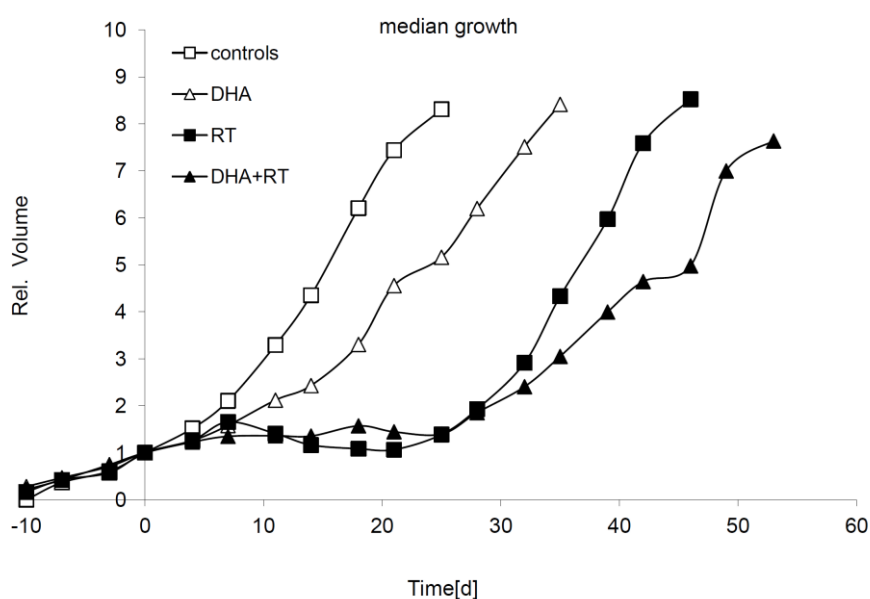
### 7.1 Supplementary data



**Fig. S1: Mcl-1 is not influenced by DHA treatment in HCT116 wt, but it is upregulated in response to loss of Bax/Bak.** HCT116 wt and HCT116 Bax/Bak<sup>-/-</sup> cells were plated and treated under normoxic or hypoxic conditions. At the 8 h time point, cells were lysed for the Western blots. Tubulin was used as the loading control and it was considered in the densitometric analysis. The experiment was performed two times and the representative blots are shown.



**Fig. S2: DHA-induced senescence is Bax/Bak independent.** HCT116 wt and Duo Bax/Bak negative cells were plated and treated with DHA under normoxic and hypoxic conditions. The cells were stained for senescence after 6 and 8 days of DHA treatment. In case of hypoxia the cells were incubated for 24 h at 0.2 % O<sub>2</sub> and then under normoxic conditions. Scale bar corresponds to 100  $\mu$ M. The experiment was done only one time.



**Fig. S3: DHA in combination with ionizing radiation induced delay in HCT116 tumor growth *in vivo*.** Human HCT116 cells were implanted to the leg of nude mice (xenograft model). When the tumors reached 100 cm<sup>3</sup>, animals were treated with 100mg/kg DHA or 2 Gy or the combination of both. Treatments took place for 5 days. In case of combination treatments, DHA was injected intraperitoneally half an hour before the ionizing radiation. In control mice, DMSO was injected (DHA was solved in DMSO). Tumor volume was measured two times a week and the experiments stopped when the tumor reached 8 fold the initial volume. The graph represents one single experiment and data from 5 mice per group.

## 7.2 Abbreviations

### A

ADP	Adenosinediphosphate
AIF	Apoptosis inducible factor
APAF1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ART	Artemisinin
Atg	AuTophagy related
ATM	Ataxia telangiectasia mutated
ATP	Adenosin-Tri-Phosphat
ATR	ATM and Rad3-related

### B

Bad	Bcl-xL/Bcl-2 associated death promotor
Bak	Bcl-2 antagonist / killer
Bax	Bcl-associated X-protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BH	Bcl-2 homology domain
Bid	Bcl-2 interacting domain
Bim	Bcl-2 interacting mediator of cell death
BSA	Bovine serum albumin

### C

CARD	Caspase recruitment domain
CBP	CREB Binding Protein
CCCP	Protonophore carbonyl cyanide m-chlorophenylhydrazone
CD95R	CD95 receptor
CD71	Transferrin reseptor
Cy2	Cyanine Dye 2

### D

DISK	Death inducing signaling complex
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
DNA-PKc	DNA-dependent protein kinase, catalytic subunit
DD	Death domain
DED	Death effector domain
DHA	Dihydroartemisinin
DSBs	DNA double strand breaks
DTT	Dithiothreitol
$\Delta\psi_m$	Mitochondrial Membrane Potential

### E

ECL	Enhanced-chemiluminescence
EDTA	Ethylen-Diamin-Tetraacetat
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EtOH	Ethanol

**F**

FACS	Fluorescence Activated Cell Sorting
FADD	Fas-Associated protein with Death Domain
FDR	Fractions of DNA released
FBS	Fetal Bovine Serum
Fig.	Figure
FITC	Fluorescein Isothiocyanat
FSC	Forward-scattered

**G**

GF	Growth Factor
GFP	Green fluorescent protein

**H**

HEPES	N-(2-Hydroxyethyl)-Piperazin-N'-2-Ethansulfonsäure
HIF-1	Hypoxia-inducible factor 1
H2AX	H2A histone family, member X
HCL	Hydrogen chloride
HRP	Horseradish peroxidase
HREs	hypoxia-response elements
Hx	Hypoxia

**I**

IgG	Immunglobulin G
-----	-----------------

**K**

KCl	Potassium chloride
-----	--------------------

**L**

LC3	Light chain 3
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**M**

Mcl-1	Myeloid cell leukemia sequence 1
mTOR	Mammalian target of rapamycin
MgCl <sub>2</sub>	Magnesium chloride

**N**

NaCl	Natriumchlorid
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NaOH	Sodium hydroxide
NF-κB	Nuclearfactor-kappa B
Neg	Negative
NT	N-terminal
Nx	Normoxia

**O**

OD	Optical density
2-OG	2-oxoglutarate

**P**

PARP	Poly (ADP-ribose) polymerase
PE	Plating efficiency
PI3k	Phosphoinositid-3-Kinasen

p53	protein 53
PBS	Phosphat-buffered Saline
PFA	Paraformaldehyde
PFGE	Pulsed-field gel electrophoresis
PHD	Proline hydroxylases
PI	Propidium Iodide
PIKKs	Phosphatidylinositol 3-kinase-related kinases
PKB	protein kinase B
PTEN	Phosphatase and Tensin homolog
PVDF	Polyvinylidene fluoride
pVHL	Product of the von Hippel–Lindau
<b>R</b>	
RNA	Ribo nucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute Medium 1640
RT	Room temperature
<b>S</b>	
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SF	Survival fraction
SSC	Side-scattered
<b>T</b>	
TBE	Tris base, boric acid and EDTA
TBS	Tris Buffered Saline
TEMED	N, N', N'-tetramethylethylenediamine
TMRE	Tetramethylrhodaminethylester-perchlorate
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris-(hydroxymethyl)-aminomethane
Tween-20	Polyoxyethylen-(20)-sorbitanmonolaurat
<b>V</b>	
pVHL	von Hippel-Lindau
VEGF	Vascular endothelial growth factor
<b>W</b>	
WST-1	Stable tetrazolium salt
Wt	Wild type
<b>X</b>	
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
XRT	Radiation Therapy
<b>Z</b>	
zVADfmk	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

Für Mengen-, Größen-, Zeit- oder andere physikalische Angaben wurden SI- (Internationales Einheitensystem) oder gesetzliche Einheiten gewählt.



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## **7.7 Curriculum Vitae**

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## 7.8 Declaration

### Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, f der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Dihydroartemisinin is a hypoxia active anticancer drug that improves the cytotoxic action of radiotherapy“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Teona Ontikatzte befürworte.

Essen, den \_\_\_\_\_

Verena Jendrossek

### Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, c und e der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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